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Endomitotic Reduplication Mechanisms in Ascites Tumors of the Mouse[1,2]

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The inconstancy of the mitotic mechanism in tumor tissue has been recognized and speculated upon since Boveri did so in his book (*1*). In comparison with the less erratic nuclear conditions in normal cells, the considerable range in the metaphase chromosome numbers of malignant cells reflects a general trend. Besides numerical fluctuations in the chromosome set, a closer study reveals the occasional appearance of structurally new chromosome types in malignant tissues.

Analysis of the mitotic behavior of a histologic "spectrum" of neoplasms in the same animal species shows that mitosis in each tumor has its own typical pattern with regard to such properties as the average amount of heterochromatin in the resting nuclei, chromosome size, and state of chromosome contraction. The same holds true for the frequency and kind of aberrations from the normal mitotic course. During serial propagation, transplantable tumors tend to reach an equilibrium between factors upsetting and maintaining uniformity through selective processes. For continuing growth, this state of equilibrium and the resulting adaptive plasticity has greater survival value than strict constancy. A neoplasm is genetically comparable with a heterogeneous clonal aggregate of asexually reproducing microorganisms; but elimination of the less viable cellular

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variants from the neoplastic population is counteracted by the genetic compatibility of the host-environment, which is not the case in parasitism.

The chromosomes of tumors, as of normal tissues in a given species, are all descended from the original zygotic set. However, tumors may diverge from the normal chromosome constitution and mitotic behavior. Some of these divergencies could be inherent in already existing nuclear differences among normal tissues and might thus precede the malignant change, while other deviations occur later. It should be stressed that chromosomal abnormalities in cancer are so multiform and haphazard that etiologic correlation is as yet impossible.

The present paper deals with a property common to all fourteen tumors studied by us, *viz.* the occurrence of cells with double or multiple the modal chromosome number typical for each growth (*2*). This polyploidy is not necessarily a euploid multiple of the basic number of chromosomes of the species in which the tumors originated. It is well known from studies of polyploidy that chromosome numbers can be increased by a variety of cytologic mechanisms. Excluding nuclear fusion, the events inducing polyploidy all represent disturbances in the relationship between chromosome reproduction and chromosome distribution to daughter cells. The chromosome number increases when distribution to daughters is omitted after one or more reproductions.

Two main types of polyploidization may be distinguished:

1) C-mitotic duplications (resembling colchicine-arrested mitoses) arise from failure of the kinetic apparatus; this leads to doubled products called "restitution-nuclei."

2) Endomitotic duplications proceed inside the nuclear membrane, and do not involve the spindle. The doubling occurs during the resting stage; and later on, when the spindle is formed, the chromosome number is already double or multiple, ready for normal distribution to daughter cells.

C-mitotic failure of the spindle is often seen in tumors. Some lymphomas, for example, are characterized by the presence of cells in which the two chromosome halves are not distributed to daughter nuclei, but are included in the same telophase nucleus. There is reason to assume that endomitosis is of greater importance in tumor growth than c-mitosis, which will be discussed in a separate paper. Endomitosis occurs regularly in many organisms. The tetraploid cells found in normal mouse tissue probably owe their origin to it. The capacity for endomitosis is thus handed down to the cancer cells by their nonmalignant precursors. Endomitotic processes need not interfere with the centrosomic reproduction cycle, while c-mitotic doublings often lead to multipolar spindles and to the immediate breakdown of the doubled chromosome number attained.

The endomitosis encountered in the present material constitutes a complex series of mechanisms falling into two main categories:

1) Endomitosis with conspicuous activity inside the nuclear membrane: chromosome contraction leading to an endometaphase and back again to a despiralized stage. This is a constant feature in some tumors, and was found occasionally in all the neoplasms surveyed. It corresponds

closely to the phenomenon for which Geitler (*3*) coined the term "endomitosis."

2) An endomitosis in which the additional chromosome reproduction is as concealed as normal chromosome reproduction during mitosis, since it takes place during the despiralized phase. Its completion can only be recognized if a mitosis follows. As the chromosomes then contract during prophase, they have become diplochromosomes or quadruple chromosomes, each centromere being in charge of 4 or 8 chromatids. Such pictures are occasionally seen in both plant and animal material. If old specialized cells re-enter mitosis, the chromosomes often turn out to have undergone double (or even multiple) reproduction. This type is also usually referred to in the literature as "endomitosis." Since both types are to be discussed here, and since they are readily distinguishable, they will be referred to as *endomitosis* and *endoreduplication* respectively. Quite possibly, transitions between them exist; one cannot distinguish between a metaphase after a true endoreduplication and a metaphase following an endomitosis in which the centromeres have failed to divide.

Materials and Methods

The present study was carried out at the Institute for Cancer Research, Philadelphia, on the tumor spectrum maintained here. The investigation includes the following mouse ascites tumors:

a) Carcinomas—TA3, 15091a, Ehrlich, Krebs 2, K2A, K2B, and K2C. The three Krebs 2 derivatives, *viz.* sublines A and B, and single-cell clone C, were established by means of microisolation, by Hauschka (*4*).

b) Sarcomas—MC1A, MC1M, S37.

c) Lymphomas—6C3HED, DBA, A#1, A#2.

Descriptions are given in Klein's papers (*5–7*) and in the survey of transplantable neoplasms prepared by Dunham and Stewart (*8*). Complete data for the two A-lymphomas have been published by Shelton (*9*).

From the chromosomal point of view the material may be grouped as follows:

Near-diploid—TA3, 6C3HED, DBA, A#2.

Near-tetraploid with conspicuous endomitotic activity in the resting nuclei—15091a, MC1A, MC1M, S37.

Near-tetraploid without such activity—A#1, Ehrlich, Krebs 2, Krebs 2 derivatives.

The chromosome studies were made in aceto-orcein stained squash preparations. In some cases, fixation of the ascites fluid in 60 percent acetic acid before pressing and staining gave superior results, especially when the ascitic fluid was viscous and took up stain.

All drawings were made with *camera lucida*. The ideogram analyses of figures 1, 2, 3, and 9 were all drawn with the same optical system in order to make the chromosome dimensions as comparable as possible. Each plate was first drawn at a magnification of 15×100. Thereafter the chromosomes were numbered and each of them was moved into the center

TABLE 1.—*Frequency distribution of chromosome numbers counted in various mouse ascites tumors**

	2x								4x																8x							
2n= 35	40	45	50	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	190	>200	Total
DBA.....2	*41*	36	2	–	1	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	84
TA3.....–	*101*	8	–	1	–	–	–	2	14	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	128
A#2.....–	4	10	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	15
6C3HED.....8	*80*	13	–	–	–	–	–	–	8	1	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	111
MC1M.....–	–	–	–	6	23	*35*	*22*	5	–	–	1	1	2	–	1	4	9	4	4	3	2	–	–	–	1	–	–	–	1	–	5	119
MC1A.....–	–	–	–	–	–	–	–	3	*23*	5	–	1	–	–	–	–	–	–	–	–	–	–	1	–	4	1	–	–	–	–	1	39
837.....–	5	4	–	–	–	–	3	4	*5*	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	2	–	–	–	–	–	–	25
15091a.....–	–	–	–	–	–	–	1	14	*24*	10	10	1	2	–	–	–	–	–	1	–	1	–	1	1	3	–	2	2	1	1	3	78
Ehrlich.....–	1	–	–	–	–	2	1	8	*29*	10	3	–	2	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	1	58
Krebs 2.....1	3	2	3	2	3	3	5	31	*61*	24	4	5	4	2	3	1	–	–	–	–	–	1	–	–	8	4	2	–	–	–	2	174
K2A.....–	–	–	–	–	1	–	2	*35*	6	–	–	–	1	–	–	–	–	–	–	–	–	–	1	3	1	–	–	–	–	–	–	50
K2B.....–	–	–	–	1	1	–	17	*64*	8	–	–	–	1	1	–	1	–	–	–	–	3	–	2	–	–	–	1	–	–	–	–	100
K2C.....–	–	–	–	–	1	–	27	*48*	2	–	–	–	–	–	–	–	–	–	–	–	2	–	1	1	–	–	–	–	–	–	–	80
Total cells.....11	235	73	6	10	30	30	78	213	181	52	18	8	12	3	5	6	9	4	6	3	9	2	6	5	19	5	5	2	2	1	12	1061

*A summary of all chromosome counts made. The exact relation between *s* and 2*s* may not be deduced from this table, since the lower number classes are inadvertently favored in the otherwise random selection of countable metaphases. The true *s*:2*s* ratios are contained in table 5. Cell numbers representing modes are italicized.

of the viewfield and outlined again with 30 × 100 magnification. The chromosomes are arranged roughly according to size. Although it is impossible to identify any individual chromosomes, those in the same size range (especially the largest and smallest ones in each ideogram) should be comparable. This is as far as we found it possible to homologize chromosomes from different plates. Anyone interested in the exact locations of individual chromosomes in the corresponding plates may obtain this information from our files.

Chromosome Numbers and the Concept of "Stemline"

It was found by Hauschka and Levan (*10*) that each of five tumors studied had a characteristic chromosome-number distribution. In some cases the modal value was close to the somatic chromosome number of the mouse ($2n=40$); in other cases it was grouped around the double of that number ($2n=4x=80$). In this respect a perfect correlation was established with the desoxypentose nucleic acid values earlier determined by Klein (*6*) for the same tumors. Some actual chromosome counts were published by Levan and Hauschka (*2*), Hauschka and Levan (*11*), and Hauschka (*4*).

All chromosome counts on an extended series of neoplasms have been collected in table 1. The data in this table are centered on classes of 5 to eliminate minor errors unavoidable in some of the less favorable tumors. The MC1M tumor, for instance, has in all fixations given very sticky chromosomes. The numbers in table 1 for this tumor are perhaps somewhat too low because of the 1 to 4 minute chromosomes that have a tendency to stick to larger chromosomes and thus escape notice. A distribution of the safest counts in this tumor is given later in this paper; its mode is somewhat to the right of that of the entire MC1M tumor in table 1. In most cases, however, the counts of table 1 are critical, the possible error seldom exceeding 1 or 2 chromosomes.

It is evident that each tumor has a rather wide range of chromosome numbers, and that this range varies considerably from tumor to tumor. Generally the diploid tumors are less variable in this respect than the tetraploid. Within the tetraploids, however, pronounced differences occur. Most striking is the difference between Krebs 2 tumor stock, which has an extremely broad distribution, and the three sublines microisolated from Krebs 2, where divergencies from the mode are very narrow.

The chromosome classes most commonly occurring often coincide with a euploid chromosome number of the mouse, either $2x$ or $4x$. Some exceptions are apparent. The A#2 tumor seems to be above $2x$, while the MC1M tumor certainly is below $4x$, as are K2A, K2B, and K2C. The chromosome class with the highest number of cells, and the adjacent classes, represent the types mainly responsible for growth and characterize the principal *stemline* of each tumor.

For convenience we will designate the most frequent chromosome number class as s, which has the following values in the material studied:

TA3, 6C3HED	40	$2x$
DBA	42	$2x+2$

A#2	44	$2x+4$
MC1M	70	$4x-10$
K2C	74	$4x-6$
K2A, K2B	75	$4x-5$
MC1A, 15091a, Ehrlich, Krebs 2	80	$4x$

It should be remembered that the tumors are not static; s may shift with time. An important fact is that s-numbers deviating from the euploid x-numbers do occur. This supports the view that the neoplastic populations have undergone genotypic changes since their origin. As the mere addition or subtraction of entire chromosomes would normally lead to genotypically unbalanced, hence less successful combinations, it may be postulated that the evolution of these tumors with aneuploid s-numbers also involves structural chromosome rearrangements. This can be observed directly in those cases where "new" chromosome types have appeared in the s-ideogram. That development takes different courses from comparable starting points is evident from the histories of the TA3 and 15091a tumors, both of which originated in mammary tissue in the same inbred mouse strain (A strain).

It is seen from table 1 that, in addition to the chromosome numbers grouped around s in each tumor, there are also numbers in entirely different regions of the distribution curve. Thus, in all tumors for which enough data have been gathered, some cells have roughly $2s$ chromosomes. This secondary mode of the distribution curve can be ascribed to chromosome doublings in the s-cells. The question arises whether the $2s$ cells are more or less permanent, maintaining themselves in the population once they have formed, or whether they constantly arise anew. This is pertinent to the question whether the stemline is comparatively simple or whether several parallel stemlines exist. If a $2s$ cell were superior in viability to the s-genotype, the cell population as a whole should gradually shift toward $2s$. Tetraploid tumors may, thus, have originated from diploid predecessors; they could on the other hand have been tetraploid from the start, the malignant transformation having happened in a normal tissue that already contained some tetraploid elements.

It is conceivable that a tumor could contain 2 separate stemlines, one having twice the chromosome complement of the other. Such a condition should be highly labile; mutations within the two stemlines would tend to favor one of them. In this connection S37 should be mentioned: its stemline is certainly $4x$; still, a certain proportion of $2x$ cells persists. In the exact counts for S37 the $\frac{1}{2}s$ fraction (table 1) is relatively too large, because these numbers can be counted more easily. In reality, only about 3 percent of the cells are diploid. It may be concluded that the diploid counts in S37 derive from nonmalignant somatic elements; or that S37 has a specific mechanism for the perpetual production of diploid cells out of the $4x$ stemline; or the diploid cells found are relics of the original population, which may have had a $2x$ stemline with sufficient competitive ability to survive as a small residue. In serial propagation of the tumors each transplant is given a new environmental start. Therefore, the more

antigenic cell types capable of vigorous growth shortly after transfer may be temporarily overgrown by competing types during the latter part of each transfer generation.

The problem of the side-by-side existence of two stemlines could be attacked experimentally by inoculating known mixtures of $2x$ and $4x$ stem-cells after microisolation from the same tumor, and subsequent population analysis.

The $2s$ fractions of the tumors arise, of course, through the more or less regularly functioning mechanisms for the new production of chromosome doubling. The frequency of $2s$ cells varies greatly in different tumors, regardless of ploidy. Among the diploid ascites tumors, for example, DBA has less than 3 percent of $2s$ cells; TA3, in some squash preparations from the most recent generations, has as many as 30 percent $2s$ cells. Perhaps TA3 is now undergoing transition toward polyploidy. The factors governing the relation between s and $2s$ in each tumor are: *a*) the frequency with which new doublings are produced, *b*) the viability of the doubled cells as measured by their ability to enter and complete new mitoses after the doubling, and *c*) the division rate of these mitoses. Evidently each tumor has established an equilibrium between these different factors, making the fraction of $2s$ cells encountered in different samplings usually quite constant. Direct demonstration of this process is furnished by clone K2C, which was critically derived from a single presumably near-tetraploid cell, and which already during its first two transplant generations showed about 1.5 percent $2s$ cells.

The sum total of all classes in table 1 has been added, although the absolute proportions of the various chromosome-number classes are, of course, determined by the arbitrary composition of our particular tumor spectrum. Still, the summary gives some information about the general tendency of the chromosome numbers to gather around euploid x numbers. Undoubtedly not only $4x$ and $8x$ are favored, but slight indications of small modes suggest themselves also at $3x$, $5x$, $6x$, and $7x$. It seems that cells which through random chromosome combinations happen to fall in the vicinity of a euploid number have selective advantages over the aneuploid classes intermediate between these numbers.

Chromosome Morphology of the Tumor Cells

In studying the chromosome morphology of each tumor, comparison with the normal tissue of origin would have been desirable. We consider this to be one of the most urgent issues of further cytologic analysis.

The only samples of chromosomes of normal tissue of the mouse that we studied were some squash preparations of whole mouse embryos, approximately 10 days *in utero*, and some squashes of testis tissue. The embryonal tissue contained an abundance of mitotic figures at all stages. Some metaphases were clear enough for a complete ideogram analysis. Of ten cells thus analyzed, nine had 40 chromosomes and one had 41. The numbers 41 and 42 were also counted occasionally in two slides containing about 150 good metaphase plates. In this latter sample two plates

were tetraploid. Aberrant numbers occur with relatively low frequency in embryo tissue. Figures 1*A* and *B* show one of the embryonal metaphases and should represent the normal somatic ideogram of the mouse. This agrees with the findings of earlier workers. All chromosomes are terminally attached; their sizes range continuously from 2.0 μ to 5.5 μ. One ideogram of a somatic interstitial cell from a testis is pictured in figures 1*C* and *D*. The chromosomes are somewhat smaller, but resemble the embryonic chromosomes in all other respects.

In figures 1*E–L* ideograms are given of representative metaphases from four diploid tumors that conform well with the chromosomes of normal tissues. Each tumor, however, has its individualistic, though sometimes rather subtle, features. The DBA lymphoma (figs. 1*E*, *F*), for instance, has unusually contracted chromosomes; the chromatids are often free from each other and the chromosomes may occupy the entire spindle equator which, in turn, extends through most of the plasma. In the TA3 tumor (figs. 1*G*, *H*) the chromosomes are freely arranged in a ring along the margin of the spindle (fig. 6*K* shows this very clearly). The spindle does not fill up the cell, allowing plenty of room for the chromosome arms to extend into the cytoplasm and rendering them more accessible to detailed study.

Similar analyses of some tetraploid tumors are given in figure 2, reproduced at the same scale as figure 1. Figures 2*A* and *B* are ideograms of lymphoma A#1, a tetraploid tumor from the same inbred mouse genotype and the same tissue as A#2 of figures 1*I* and *J*. Only a few cells in this tumor have been analyzed because of generally crowded chromosomes. The latter are somewhat smaller than in the diploid lymphoma A#2, and the contraction is generally greater, which makes this tumor a difficult cytologic object. Figure 2*C* is from the tetraploid rhabdomyosarcoma, MC1A. Figures 2*E–H* should be compared with figures 9*A–D* since they are representative of the Krebs 2 tumor and its sublines which, together with the Ehrlich ascites tumor, are the cytologically most favorable ascites material. The cells are large in relation to chromosome size, allowing the plates ample space to spread.

The morphology of the chromosomes in the tetraploid tumors is not noticeably different from the diploids, with the possible exception of the average size which is somewhat smaller in the former. This is reminiscent of analogous conditions reported from autotetraploid plants.

Chromosomes with detectable structural deviations have been most often encountered in tetraploid tumors, probably because the more plastic polyploid genotype permits greater tolerance of structural variations. The following two types of changes can be detected: the appearance of bi-armed chromosomes in the otherwise telocentric set, such as those described by Makino (*12*) for several rat tumors; size changes of sufficient magnitude to put individual chromosomes significantly outside the normal size range.

Seemingly bi-armed chromosomes requiring further study were often seen in the 15091a tumor, but in no other tumors. Chromosomes with a

small head in addition to the normal longer arm appear now and then. The ideograms of TA3 (figs. 1*G*, *H*), MC1A (figs. 2*C*, *D*), and K2C (figs. 2*G*, *H*) tumors contain such anomalies. The frequent anaphase disturbances are indications of occasional variation in chromosome structure.

Telocentric chromosomes considerably above and below the normal size range are typical of certain tetraploid tumors, where they appear in the stemlines, as judged from their high frequency. The MC1M and MC1A tumors and S37 have such chromosomes. In MC1M, one very large chromosome (the first in fig. 3*F*, at 6 o'clock in fig. 3*G*) and a fluctuating number of very small chromosomes are regularly present. The latter, ranging in number from one to four, measure about half the length of the smallest ordinary chromosome of normal mouse tissue (figs. 3*F–I*). Similar small chromosomes have been reported recently by Bayreuther (*13*) in his hyperdiploid line of the Ehrlich ascites tumor. In MC1A (figs. 2*C*, *D*) and S37 (figs. 5*M–V*) these minute chromosomes occur regularly; in K2 (figs. 9*E*, *F*), rarely.

The presence of "new" chromosomes in the stemline of certain tumors, *e.g.* MC1M, is perhaps a more tangible expression of evolutionary processes than are simple numerical variations. Both types of change entail gross shifts in hereditary potential and broadened adaptability.

Endomitosis

The foregoing survey of the usual chromosome conditions in the neoplastic material under study will serve as a basis for discussion of endomitotic mechanisms, as defined by Geitler (*3*). The endomitosis to be described here is essentially the same kind as depicted by Biesele, Poyner, and Painter [(*14*), figs. 45, 52, 53] in other tumors of the mouse.

Strongly contracted, metaphase-like chromosomes lacking spindle-orientation, and evenly situated inside a nuclear membrane, were occasionally seen in all the tumors studied. Interpretation of these stages was difficult until one tumor was found in which such phenomena were the rule. Most "resting nuclei" were in one or another stage of endomitosis and exhibited a great variety of chromosomal structures. In place of the uniform appearance of normal resting stages, these nuclei by their great morphologic diversity convey the impression of pronounced chromosomal activity within the nuclear membrane.

Endomitosis acts regularly in the growth of MC1A, MC1M, S37, and 15091a tumors. MC1M will serve as a typical example; certain stages of endomitosis will be demonstrated by other neoplasms. The normal mitotic cycle of MC1M is pictured in figure 3. Figures 3*A*, *B*, *C* represent typical metaphase plates of s, $2s$, and $4s$ cells respectively; figures 3*D* and *E* show normal prophases. These prophases are readily distinguishable from endomitotic stages by their relic coiling and chromosome banding.

Since metaphase chromosome numbers are a clue to the frequency of endomitosis, distribution of the 51 most critical counts in the MC1M tumor is summarized in table 2, which gives the frequency ratio 39:11:1 for s to $2s$ to $4s$. In 122 metaphases from 9 slides in which only approxi-

mate estimates of chromosome number were made and classified as multiples of *s*, the same ratio was 76:38:8. Since endomitosis is certainly the prevalent doubling mechanism in MC1M, these values are fairly representative of the cytologic consequences of endomitosis. Whether they give a reliable picture of the real composition of the cell population depends, of course, on the rate at which the several ploidy classes enter mitosis.

TABLE 2.—*Distribution of chromosome counts in the MC1M sarcoma ascites*

Multiples of *s*	*s*					2*s*						4*s*
Chromosome number	60	65	70	75	95	115	120	125	130	135	140	265
Number of cells	1	16	17	4	1	1	1	3	4	1	1	1

In table 3, an attempt was made to classify the cells of the MC1M tumor by nuclear size into 3 groups, each subdivided according to the number of nucleoli per nucleus. The distribution trend is roughly the same as in the ploidy classification. The average number of nucleoli is higher in the medium-sized than in the stemline nuclei, although the maximum number is about the same. In cases of endomitosis, as well as in endoreduplication, an increase in number of nucleoli is unlikely until after the doubled cells have gone through a mitotic division during which the nucleolus-organizing segments of the daughter chromosomes are separated from each other.

TABLE 3.—*Classification of MC1M cells according to nuclear size and number of nucleoli*

Nuclear size	Stemline size (*s*)						Medium size (2*s*)							Large size (4*s*)						
Number of nucleoli	1	2	3	4	5	6	1	2	3	4	5	6	7	2	3	4	5	6	7
Number of cells	80	65	36	8	1	1	13	15	12	14	8	3	2	6	2	1	1	—	1
Total cells	191						67							11						

In this connection it should be pointed out that the rate of endomitotic duplication in a tumor might exceed that of normal mitotic activity, so that the majority of cells would be 2*s*, 4*s*, or 8*s*. Under such conditions, desoxyribonucleic acid determination without support of cytologic data would, of course, yield no information as to the chromosome number of the stemline, but would merely reflect the average chromosome number of the entire cell population tested.

The resting nuclei of the MC1M tumor, and other tumors showing regular endomitosis, contain numerous heterochromatic bodies. Even during the most despiralized resting stage, the number of heterochromatic bodies corresponds approximately to the chromosome number of the cell (figs. 4*A*, *B*), which suggests that each chromosome is represented by a heterochromatic region. There are pronounced differences between various neoplasms in the amount of heterochromatin seen in the resting

nuclei. Figures 4*K*, *L* show the situation in the TA3 tumor, where a few comparatively large heterochromatic bodies are found, while figure 5*A* depicts a typical resting nucleus of Krebs 2 tumor, which is characterized by several small bodies. In all tumors, as in normal somatic cells, the largest heterochromatic bodies are attached to the nucleoli, as pictured by Biesele, Poyner, and Painter [(*14*), figs. 35–39, p. 37]. A resting nucleus with double *s*-number is shown in figure 4*C*. It is noteworthy that those tumors that have regular endomitosis also exhibit the largest amount of heterochromatin during the resting stage.

We have failed to recognize any endomitotic prophase other than a general growth of the heterochromatic bodies, which sometimes fill the entire nucleus (figs. 4*D*, *E*). They are irregularly shaped, but many of them have about the same proportions as the chromosomes. During this growth, the chromatic granules in the nuclear sap around the heterochromatin disappear; when "endometaphase" is reached the background of the chromosomes is quite clear. Figures 4*G* and *H* are representative of this stage. The chromosomes now have become more distinct; they are often clearly divided and their centromeric ends may be recognized. At full endometaphase, the nucleoli are small or have disappeared, and the number of chromosomes can easily be estimated. In figures 4*G* and *H* it is about 70; in 4*I* and *J* it is around 130.

The endomitotic sequence in the MC1M tumor could be followed only to endometaphase. No clear separation of the chromatids was ever seen, probably because of "stickiness" which was a constant obstacle in this tumor. The missing stages have been observed clearly in other ascites tumors, however. In TA3, where endomitosis is exceptional, the very clear endometaphase of figure 4*M* was observed. Its chromosomes resemble ordinary chromosomes much more than in MC1M; contraction is, however, more pronounced than during normal metaphase. The clear presence of a nuclear membrane and the absence of spindle orientation confirm that this picture belongs to endomitosis. Similar exceptional figures were now and then encountered in Krebs 2. Figures 5*B–D* represent a few such instances. Figure 5*B* is a stage like the one just described for TA3. In figure 5*C* the nucleus had been severely squashed so that the chromosomes were spread out in one plane, giving the superficial impression of the "ball-metaphase" frequent in Krebs 2. It differs in several respects, however: the chromosomes are smaller and more completely separated than in ball-metaphase. In the latter, the chromatids normally touch each other at both ends of the chromosome while the middle of the chromosome is vacuolized. Further, the chromosomes in figure 5*C* are not held by a spindle and appear to represent an endoanaphase. Such anaphases are very rare, since the stainability is usually lost before the chromosome halves fall apart, as in figure 5*D*, which shows a segment of an endotelophase of a large Krebs 2 tumor cell with about 150 clearly divided chromosomes, still held together at the centromeres.

Sarcoma 37 furnished good examples of the later stages of endomitosis, which starts out as already described for the MC1M sarcoma with growth

of the heterochromatic bodies in the resting nucleus (fig. 5*E*). These bodies characteristically have a dense center surrounded by radial chromatic outgrowths, recalling the structure of lampbrush chromosomes. In figures 5*F–I*, the development of the usually smaller and less clearly defined endomitotic metaphase chromosomes may be compared with ordinary metaphase chromosomes (figs. 5*M*, *N*).

The later stages of endomitosis in S37 were sometimes very distinct. They showed that the metaphase chromosomes gradually lost their stainability, often disappearing without chromatid separation. Now and then the chromatids were far apart (figs. 5*J–L*), but still converged at their centromeric ends. The daughter chromatids were frequently enveloped in a cloud of chromatic material.

Since no mitoses in the tumors with regular endomitosis had their chromosomes arranged in pairs or formed diplochromosomes, it is apparent that the daughter chromatids of endomitosis are able to move apart before reappearance in the next mitosis. This is basically different from endoreduplication, where the chromosomes never move apart but persist as diplochromosomes until the following metaphase. The endomitosis thus has a typical anaphase and telophase, although telophase often starts before the anaphase separation of the chromatids. The chromosomes of these stages are always considerably smaller than those of corresponding phases in ordinary mitosis (*cf.* figs. 5*J–L* and 5*V*, *W*).

It should be stressed that the account of endomitosis here given is to be regarded more as a sampling of stages than as a complete developmental series. Seriation is often difficult to distinguish, which may mean that endomitosis is less strict in its sequence than normal mitosis. This might be expected, since endomitosis is not dependent on close timing between chromosomal changes and spindle behavior. The important role of endomitosis in the cellular growth of certain normal plant and animal tissues raises the interesting question of whether endomitosis as a constant feature in the life cycle is acquired by certain tumors after their origin, or whether it has been "inherited" from their nonmalignant precursors. This can only be decided through cytologic comparison with homologous material.

Endoreduplication

In this type of internal doubling, the extra division of the chromosomes takes place during the resting stage without any mitosis-like manifestations. The term "masked endomitosis" was used by Resch (*15*) to distinguish it from the "open endomitosis." It is connected with cellular volume growth in many plants, and gives rise to the polysomaty of older parts of roots often commented upon. Polytene chromosomes result if it repeats itself several times in the resting stage. Except in instances where the polyteny takes extreme form, as in the salivary-gland chromosomes of Diptera, the endoreduplication will first be visible during prophase of the subsequent mitosis. Usually it makes itself apparent through "paired" chromosomes or quartets. Sometimes the centromeres do not divide

until after the arrangement of the chromosomes on the equatorial plate, which will then contain diplochromosomes. This behavior was described for *Allium cepa* root cortex by Levan (*16*). The endoreduplication of cancer cells agrees fairly well with the corresponding phenomenon in *Allium cepa*. Capacity for double or multiple reproduction during resting stage seems to be a general property of the chromosome thread.

The diagnostic feature of endoreduplication is the presence during prophase and metaphase of diplochromosomes. These fall apart into the double number of ordinary chromosomes before anaphase. After separation of the diplochromosomes, the course of mitosis is normal, the end result being two daughter nuclei with doubled chromosome sets. Certain deviations from this typical behavior will be mentioned under the description of endoreduplication in individual tumors.

Endoreduplication in Diploid Tumors

The TA3 tumor will serve as the typical example of endoreduplication. It is seen from table 1 that this tumor has a diploid stemline with fairly frequent 2*s* numbers. As an instance of chromosome counts underlying the values of table 1, the distribution found in a sample grown in strain A mice is given in table 4. The frequency of 2*s* cells is 12 percent in this case; in other samples it varied between 10 and 30 percent. This is the highest value for 2*s* obtained in any tumor except those with regular endomitosis, where the 2*s* value may sometimes be higher. In TA3 tumors, most of the doublings are induced by endoreduplication, a high incidence of which can be directly observed during prophase-metaphase. The frequencies of doubled chromosome numbers and unquestionably identified endoreduplication are listed in table 5. The endoreduplication value recorded is a minimal percentage, since endoreduplication is more doubtful after separation of the diplochromosomes during late metaphase. It may be concluded that the frequency of endoreduplication in this tumor is directly responsible for about half of the observed 2*s* metaphases, which means that each 2*s* cell divides on an average only once after the doubling, provided the rate of division is the same in all 2*s* mitoses.

TABLE 4.—*Chromosome counts in one of several similar samples of TA3 mammary adenocarcinoma ascites*

Multiples of *s*	*s*						2*s*			
Chromosome number	38	39	40	41	42	54	75	78	79	80
Number of cells	1	1	23	15	3	1	1	1	1	3

Figure 6 represents the course of endoreduplication in the TA3 tumor. The appearance of the normal chromosomes at metaphase in one *s* cell and one 2*s* cell is shown in figures 6*K* and *L*, which should be compared with the diplochromosomes of the other pictures. Figure 6*J* is an *s* cell with considerable chromosome contraction. The endoreduplication pro-

phase of figure 6*A* is distinguishable from normal prophases, for instance figure 3*D*. Each prophase chromosome of figure 6*A* consists of 2 chromosomes joined at the centromeric region. Since all the chromosomes are clearly divided, the centromere holds together four chromatids instead of two as in ordinary chromosomes. In figure 6*A* the components of each diplochromosome have become free from each other except for their centromeric portion. They form structures resembling medially attached chromosomes. A similar cell was pictured by Biesele, Poyner, and Painter [(*14*), fig. 22] and interpreted as endoreduplication. Sometimes the constituents of each diplochromosome hold together throughout prophase along their entire length. This is the case in the somewhat more advanced prophase of figure 6*B*. The chromosome contraction may become very tight before the nuclear membrane disappears, as in figure 6C. Eventually the nuclear membrane dissolves and the centromeres respond to the spindle. The chromosomes are still connected as diplochromosomes (fig. 6*D*), with their centromeres oriented on the spindle like typical mitotic centromeres. If figures 6*D* and *K* are compared, the same general pattern of orientation on the spindle is evident.

TABLE 5.—*Endoreduplication in mouse ascites tumors*

Tumor	No. of samples	No. of metaphases studied	Percent metaphases 2*s*, 4*s*, >4*s*	Percent endoreduplication	Sampling range for percent endoreduplication
TA3	4	252	18.6	8.7	6.0–12.3
6C3HED	4	536	3.4	0	0
DBA	4	558	0.9	0	0
MC1A	2	285	18.6	3.5	2.5–4.6
S37	3	127	7.6	1.7	0–4.8
15091a	4	193	41.4	20.2	15.8–28.2
Ehrlich	3	1554	2.8	0.4	0.2–0.7
Krebs 2 stock	3	3288	2.9	0.2	0–0.4
K2C	2	742	1.8	0.1	0–0.2

The first division of the centromeres takes place now and in some cases a very striking picture is seen: for a short period the centromeres of the two halves of a diplochromosome are directed toward opposite poles and are, it seems, actually pulled toward them, while the main bodies of the diplochromosomes still hold together. Especially in side view, the diplochromosomes may during this stage strikingly resemble bivalents. This is a very transient condition, although signs of it may persist for some time afterward. This behavior is evident in several of the diplo-pairs of figure 6*E*, in which the centromeric regions of the chromosomes of a diplo-pair are clearly bent away from each other. For a short period, the centromeres behave like meiotic centromeres.

No sure instances were seen of entire chromosomes being transported

to the poles after the "first" division of diplochromosomes. It has been noticed in exceptional cases that anaphase chromosomes were arranged in pairs within the anaphase groups, suggesting movement of sister chromatids to the poles as units. Prophases like figure 6*A* where the halves of each diplochromosome are entirely free from each other (except for the centromeres), the arms showing no relational spiralization, may mean that the separation of these chromosomes has occurred before the resting stage. Such would be the case if entire chromosomes had separated at the preceding anaphase. Bearing in mind the possibility of exceptional mechanisms, it may be stated that, typically, the diplochromosomes divide on the plate and that each chromosome thereafter behaves as a normal mitotic chromosome. In the terminology generally employed for describing centromeric behavior, the centromeres of the diplochromosomes are at first auto-oriented; for a short period immediately following their first division they are co-oriented, however. Then they rapidly respond again as mitotic centromeres and become auto-oriented on the plate. In figure 6*E* each centromere is clearly pulled out toward both poles.

Another interpretation, for which we are indebted to Dr. Östergren (oral communication), is the following. During the congression into the equatorial plate each diplochromosome oscillates to and fro in the two poleward directions, a type of movement known to be characteristic of chromosomes at the metaphase plate formation. If the first division of the centromeres has already taken place, the two partners of each diplochromosome may be expected to move independently of each other. Accordingly, some diplochromosomes should have both centromeres pulled toward the same pole, while some should have them pulled in opposite directions. This latter case would give an impression of co-orientation.

After the separation of the diplochromosomes, the chromosomes are still arranged in pairs for some time (fig. 6*F*), but this pattern is now gradually blurred out. In figure 6*G* it is still evident which chromosomes are derived from the same diplochromosome; their mutual arrangement is, however, changed, and soon this plate would no longer have been recognizable as belonging to a first-generation cell of endoreduplication.

Figure 6*H* is a somewhat deviating type of endoreduplication. Its chromosomes may be called "parcel" chromosomes, since they are so bunched together that all four chromatids of each diplochromosome touch each other along a central line like the four pollen grains in a tetrad. This type is often associated with comparatively strong chromosome contraction and pronounced stickiness. Ordinary chromosomes may take on a similar appearance, as in figure 6*I*, which has unusually large chromosomes, without any indication of quadruple structure.

In the two other diploid tumors (6C3HED and DBA) that were studied enough to give some indication as to the occurrence of duplication mechanisms, endoreduplication has not been encountered, and if it occurs at all it is certainly much rarer than in the TA3 tumor (table 5).

Endoreduplication in Tetraploid Tumors

(a) *MC1M tumor.*—Although endoreduplication plays a relatively insignificant role as compared with endomitosis in this tumor, several instances of endoreduplication were recorded. Figure 7*A* is an early diplophase in which the quadruple structure of the diplochromosomes was very distinct. The relational spiral of the two chromosomes was almost dissolved, but the chromatids of each chromosome were involved in an often rather tight spiral (fig. 7*B*). Figure 7*C* is a later prophase, wherein the chromosomes have become more stainable and the split within each chromosome is no longer visible. Some of the diplochromosomes seem not to be held together at the centromeres. The appearance of this prophase is different from other prophases seen in endoreduplication; it clearly belongs to the endoreduplication type of mitosis, however. Figure 7*D* is an endoreduplication metaphase. The structurally deviating chromosomes characteristic of the MC1M tumor, both the large one and 2 minute ones, are evident. In figure 7*E* details of some diplochromosomes have been drawn separately.

(b) *MC1A tumor.*—Besides the 39 exact chromosome counts in table 1, 285 counts of roughly determinable metaphases are included in the analysis of endoreduplication in table 5. Figure 7*F* shows an endoreduplication metaphase of the MC1A rhabdomyosarcoma. Not all of the ±80 diplochromosomes in this particular plate were drawn.

(c) *Sarcoma 37.*—As seen in table 1 the modal *s* value for this sarcoma is 4*x*. About 2.9 percent of the cells have ½*s* numbers, while 7.6 percent were 2*s* or 4*s* (table 5).

One of the endoreduplication metaphases observed is pictured in figure 7*G*. It deviates somewhat from the usual endoreduplication type. The 4 chromatids of each diplochromosome generally touch each other only in the centromeric region. This could be the metaphase following a prophase like the one of figure 6*A* or 8*A*. It also might be derived from an ordinary endoreduplication metaphase by strong chromosomal contraction. If the chromosomes still are kept together at the centromeres, such contraction would tend to open up the diplochromosomes by elastic repulsion, as visualized by Östergren (*17*).

(d) *15091a tumor.*—This spindle-cell carcinoma is one of the most interesting ascites tumors from the viewpoint of endoreduplication. It has a high frequency of both endomitosis and endoreduplication, the latter ranging from 16 to 28 percent in four different samples. The percentage of double reduplications (4*s* or higher) is unusually high (6.7 percent) and has been added to the 34.7 percent 2*s* value in the fourth column of table 5.

In the 15091a tumor several undoubted endoreduplication metaphases with quadruple chromosomes have been observed, *i.e.* eight chromatids joined by one centromere (figs. 7*I*, *H*). Evidently the chromosome threads have undergone two extra divisions during the preceding resting stage.

So far, the material has been too scanty for analysis of the entire mitotic cycle of the quadruple chromosomes. They were, however, arranged in a

normal equatorial plate. Often, two quadruplo-chromosomes were joined together at their centromeres and, thus, may represent reduplications of the regularly observed V-chromosomes in this particular tumor (see above).

(e) The *Ehrlich carcinoma* has a constant but low frequency of endoreduplication (fig. 8*B*). The 0.4 percent value in table 5 may not be high enough to account for the numerous cells with doubled chromosomes and especially for the giant cells characteristic of Ehrlich as well as Krebs 2 tumors. Failure of cell separation and fusion of nuclei seem to play a part in the development of these high *s* cells. Chromosome numbers as high as 400 to 500 were not rare (fig. 11), the record being the endoreduplication metaphase of figure 8*G* in which over 1,000 diplochromosomes were counted.

In the endoreduplication metaphase of figure 8*B*, also photographed as figure 10*A*, a few chromosomes were less stained than the rest. They still retained a relational spiral of the second order, which had almost disappeared in the normally contracted chromosomes.

(f) The *Krebs 2 tumor* is cytologically comparable with the Ehrlich ascites tumor in many respects: it is highly favorable material for analysis of variations in chromosomal type and contains a similar proportion of nuclei with ball-chromosomes, giant cells, cells with weakly spiralized chromosomes, and endoreduplication metaphases (table 5).

The clear detail of endoreduplication in Krebs 2 is evident from figures 8 and 9. Because of the low endoreduplication frequency, it has been impossible to rely upon the Krebs 2 tumor for all stages of endoreduplication, prophases being especially rare. The prophase in figure 8*A* is of the same type as figure 6*A*. An early endoreduplication metaphase with diplochromosomes still hanging together is shown in figure 8*C*, while figure 8*D* gives a slightly later stage, most of the diplochromosomes having separated. A similar stage is analyzed in figures 9*E* and *F*, side by side with two normal metaphases and ideograms drawn under identical conditions (figs. 9*A–D*). The two normal cells are intended to show the variation in chromosome contraction at full metaphase in Krebs 2 tumor. The upper cell has extremely long chromosomes, while the lower cell has normally contracted chromosomes. The diplochromosomes of figure 9*E* correspond with the latter in degree of contraction, but their volumes are decidedly smaller. The morphology of the diplochromosomes and the arrangement of their chromatids were quite distinct in this cell. Structures similar to chiasmata are evident (fig. 9*F*); these appear to depend on the opening up of loops of the first and second order in different parts of the same diplochromosome.

Figure 8*E*, photographed in figure 10*B*, is an endoreduplication metaphase of the same kind as described above for Sarcoma 37 (fig. 7*G*). The chromosomes form quartets, each corresponding to one diplochromosome. Figures 8*F–H* represent a Krebs 2 giant tumor cell. Cells of such dimension (150 to 200 μ in diameter) are not infrequent but they are rarely seen in mitosis. Figure 8*F* shows the size relationships between cytoplasm and chromosome plate. It should be remembered that this

cell has been flattened out in preparing the slide; several chromosomes were thereby displaced from the plate into the cytoplasm. Because of multilayered crowding of chromosomes in the equatorial plate, a detailed drawing was not feasible and only general appearance was aimed at in figure 8*G*. It became apparent that all the units in the plate were really diplochromosomes (fig. 8*H*). These seemed in addition to form larger aggregates, bundles of 4, 8, or perhaps even 16 diplochromosomes. Especially in regions of the plate where chromosomes could be recognized by their small or large size, clumps of similar chromosomes were closely adjacent. This plate, therefore, probably represents an endoreduplication of high order, the chromosomes having undergone 5 or more divisions in the preceding resting stage. Such endoreduplications would show up only as a gradual growth in volume of the resting nucleus and of the cell, and would explain why mitoses are so rarely met with in the giant cells (only 3 cases in several hundred slides like fig. 11*B*).

(g) *Krebs 2 tumor derivatives.*—In sublines K2A and K2B which were established by inoculation of one to four Krebs 2 cells (*4*) it could still be claimed that the occurrence of both *s* and 2*s* cells was due to implantation of both types; but in K2C, which is a critical single-cell clone, any cells with deviating *s* number must have been derived from the progeny of one original cell.

As is seen from table 1, all three Krebs 2 tumor derivatives contained a certain number of 2*s* cells in addition to the more numerous *s* cells. Only the values for K2C can be used for estimation of the actual frequency of 2*s* cells, since in K2A and K2B the most countable cells were selected. The proportions of 2*s* metaphases and the percent of endoreduplication are given in table 5, the frequency of 2*s* being of about the same order of magnitude as in the Krebs 2 stock tumor. Thus, K2C appears to have restituted the endoreduplication conditions in the original population. Cell sizes of K2C were, however, much more uniform than in Krebs 2 stock and the frequency of giant cells was very low. This would not show up in the chromosome number counts since the giant cells rarely enter mitosis. Only one absolutely certain case of endoreduplication metaphase was encountered in K2C, making the percent frequency 0.13, which is quite comparable with corresponding values in both Krebs 2 and Ehrlich.

The finding of the endoreduplication mechanism already in the first critical single-cell derivative of Krebs 2 is highly interesting. It may, of course, be claimed that the ability to perform this cytologic trick is inherent in only one of several possible stemline constituents of K2 tumor. However, the fact that the first single-cell clone extracted from the K2 tumor turns out to have this ability would make it seem more probable that a mixture of stemlines, all averaging the same genotype, is responsible for the propagation of the tumor. Since the two sublines so far extracted both have a modal chromosome number of 73 to 75, they probably represent the most viable region of the Krebs 2 population curve, and

therefore share the typical properties of the K2 genotype, including the capacity for endoreduplication.

Conclusions and Summary

Only through comparative study of a broad histologic "spectrum" comprising various neoplastic types, available in the cytologically favorable ascites form, can critical information be gained as to the significance of mitotic and chromosomal irregularities in malignant growth. The data here reported for eleven mouse ascites tumors, two sublines derived from not more than four cells, and one single-cell clonal derivative permit no etiologic conclusions, but reveal two general trends, *viz.: fluctuations of chromosome numbers* are considerably wider in transplantable tumors than in the normal mouse tissues investigated both here and by Boothroyd and Walker (*18*). The *concept of stemline cells*, which has been justifiably questioned by Yoshida (*19*) on the basis of his experience with a rather uniform diploid rat sarcoma, is a reality supportable both statistically (table 1) and experimentally through isolation of single cells. Each tumor is characterized by a stemline ideogram which may be numerically and/or structurally distinct from the ideograms of other tumors, as well as from the somatic chromosome set of the mouse.

The most frequent classes of chromosome number in about half our ascites tumor material had aneuploid numbers, either hyperdiploid or hypotetraploid. Even in the cases, such as the Krebs 2 tumor, where the mode lies at $2n=80$, $2n$ does not necessarily equal $4x$, which is to say that polyploidy is perhaps not a euploid multiple of the basic $x=20$. This possibility is strengthened by the predominantly hypotetraploid counts in all three Krebs 2 tumor derivatives. Since Makino's (*12*) analysis of the Yoshida rat sarcoma showed a hypodiploid mode and Bayreuther (*13*) established the hyperdiploid character of his line of the Ehrlich mouse carcinoma, the problem of aneuploidy in the stem-cells of tumors becomes an important one and calls for exact counts in spontaneous tumors and isolation of stemlines therefrom.

Investigation of chromosome numbers and morphology formed the necessary background for the determination of the relative frequencies of endomitosis and endoreduplication and the role played by these aberrant mitotic mechanisms in the propagation of each neoplasm. In summary, the most relevant cytologic findings in our ascites tumor material are:

1) Chromosome numbers vary considerably, with modal values for individual neoplasms at diploid (40), hyperdiploid, hypotetraploid, and tetraploid (80).

2) Occasional giant cells with 402, 522, and over 1,000 chromosomes have been encountered.

3) Structurally "new" chromosome types, including metacentric units and one to four minutes, occur in the ideograms of some ascites cells but not of others.

4) Normal mitosis, and two forms of endomitosis (endomitosis and endoreduplication), are described and pictured in detail.

5) Evidence for the stemline concept is seen both in the distribution patterns of chromosome numbers and in the experimental extraction of distinct sublines from the most viable region of the Krebs 2 tumor population curve.

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PLATES

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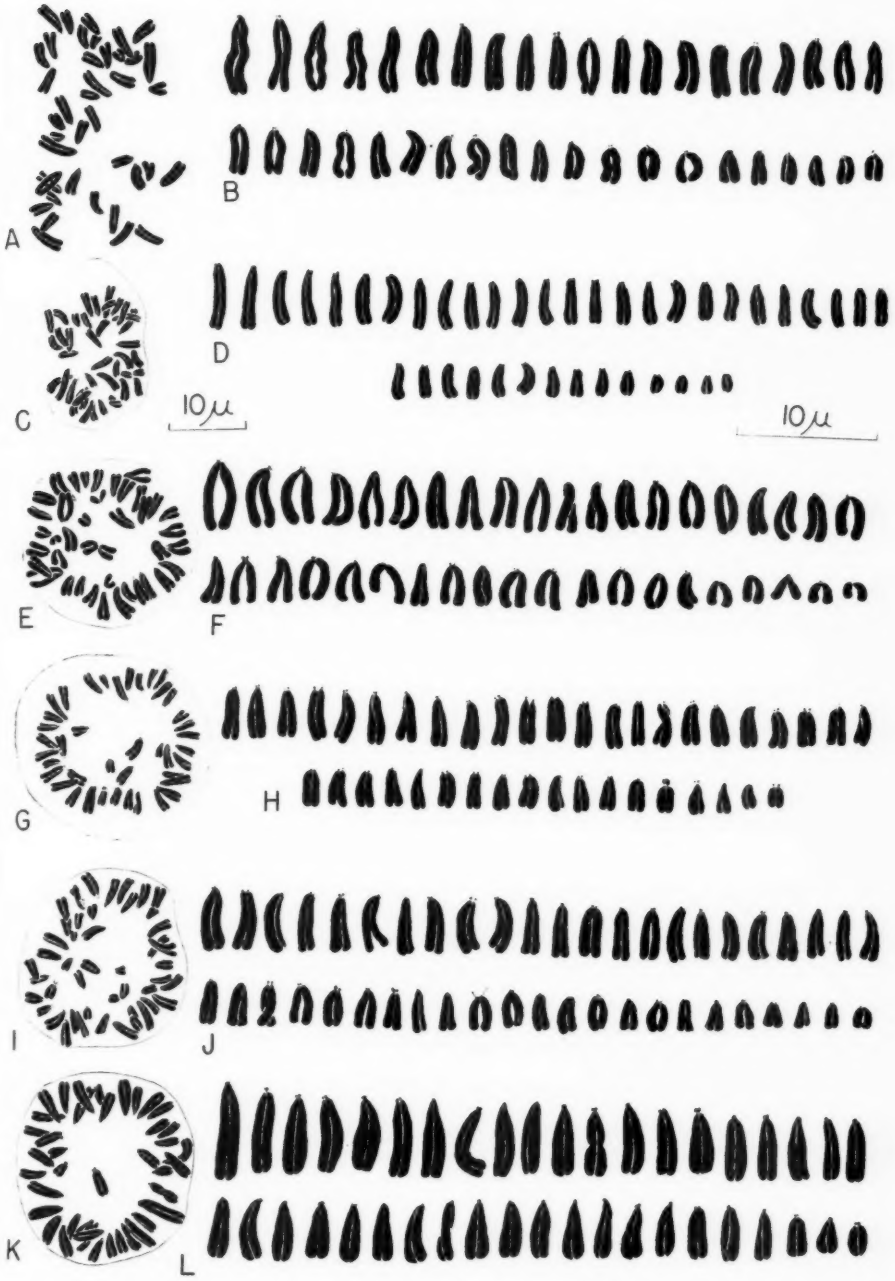
FIGURE 1.—Ideogram analyses.
A,B: mouse embryonic cell.
C,D: interstitial cell from mouse testis.
E,F: DBA lymphoma.
G,H: TA3 adenocarcinoma.
I,J: A#2 lymphoma.
K,L: 6C3HED lymphosarcoma.
A, C, E, G, I, K × 1,150; *B, D, F, H, J, L* × 2,100



Levan and Hauschka, figure 1

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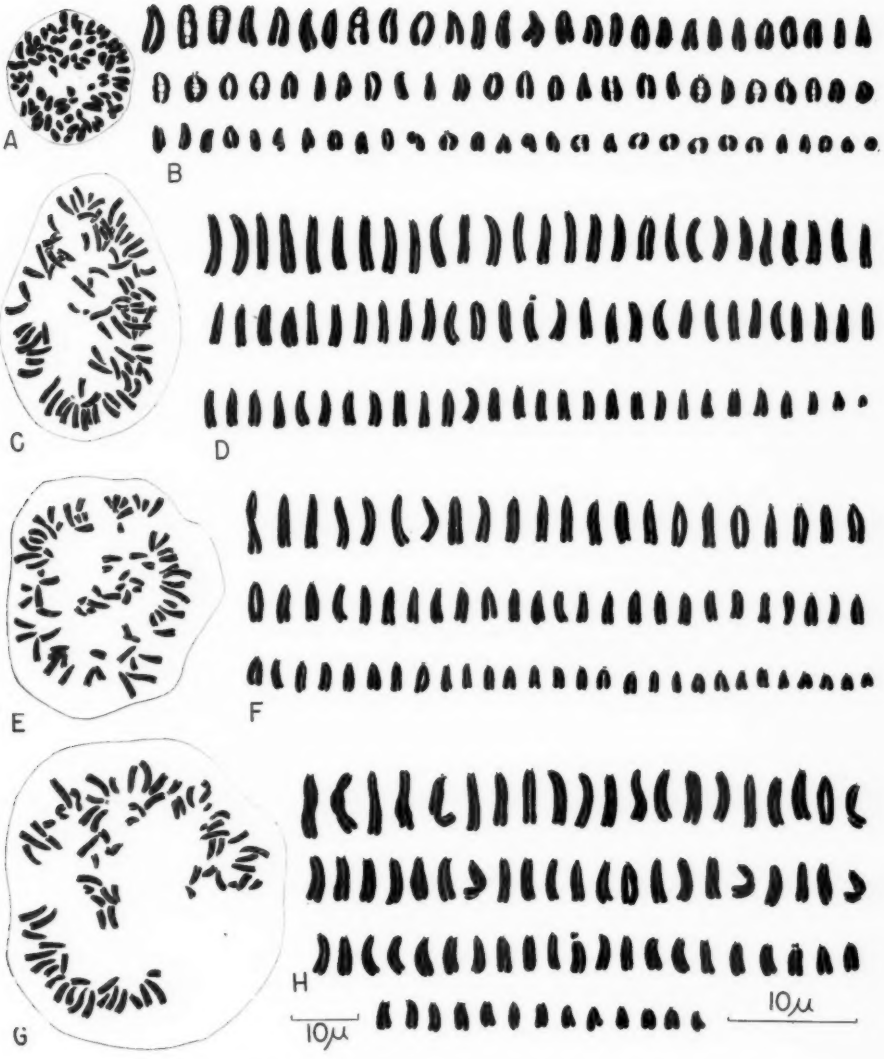
FIGURE 2.—Ideogram analyses.
A,B: A#1 lymphoma.
C,D: MC1A rhabdomyosarcoma.
E,F: Krebs 2 subline.
G,H: Krebs 2 clone C.
A, C, E, G × 1,000; *B, D, F, H* × 1,900



Levan and Hauschka, figure 2

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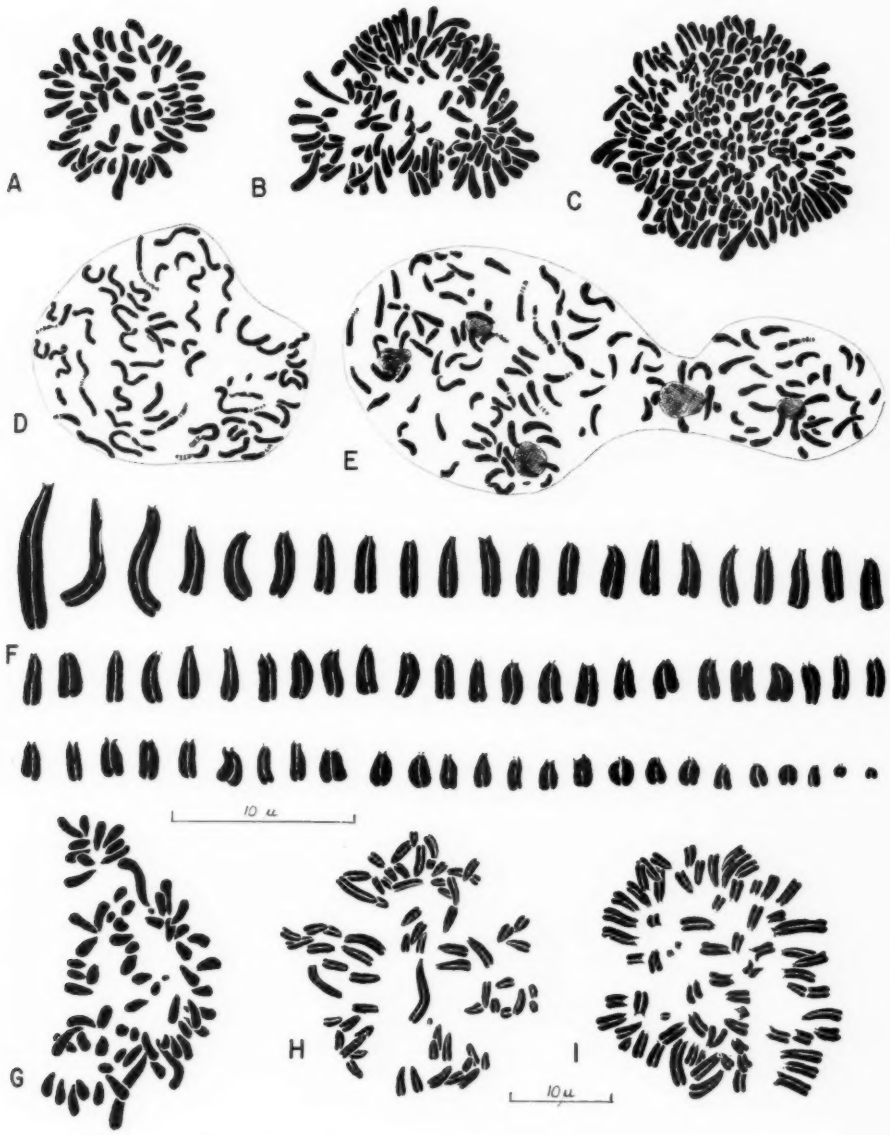
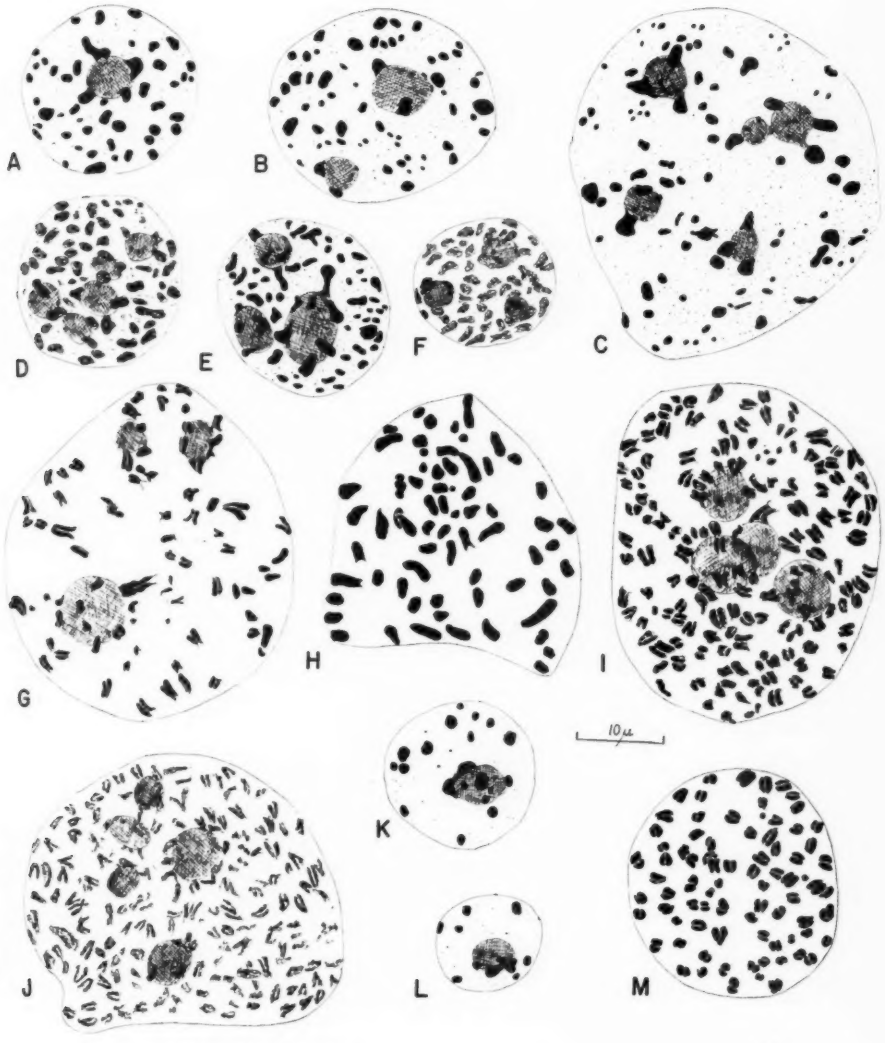
FIGURE 3.—MC1M rhabdomyosarcoma.
A–C: metaphases with *s*, 2*s*, and 4*s* chromosome numbers, respectively.
D,E: prophases with *s* and 2*s* chromosome numbers, respectively.
F: ideogram analysis of the metaphase plate of *G*.
G–I: three metaphase plates.
F × 2,750; all others × 1,500



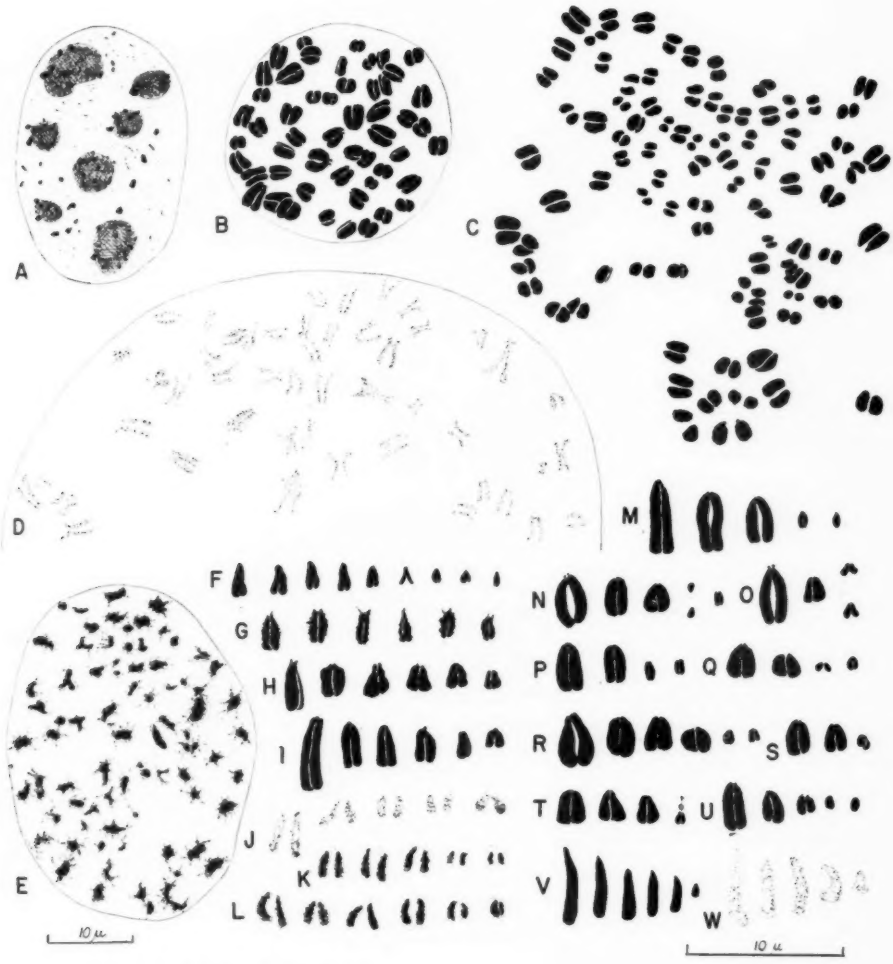
Levan and Hauschka, figure 3

FIGURE 4.
A–J: MC1M rhabdomyosarcoma.
A–C: resting nuclei.
D–F: growth of heterochromatic bodies in nuclei, preceding endomitosis.
G–J: endometaphases.
K–M: TA3 adenocarcinoma.
K,L,: resting nuclei.
M: endometaphase.
× 1,300



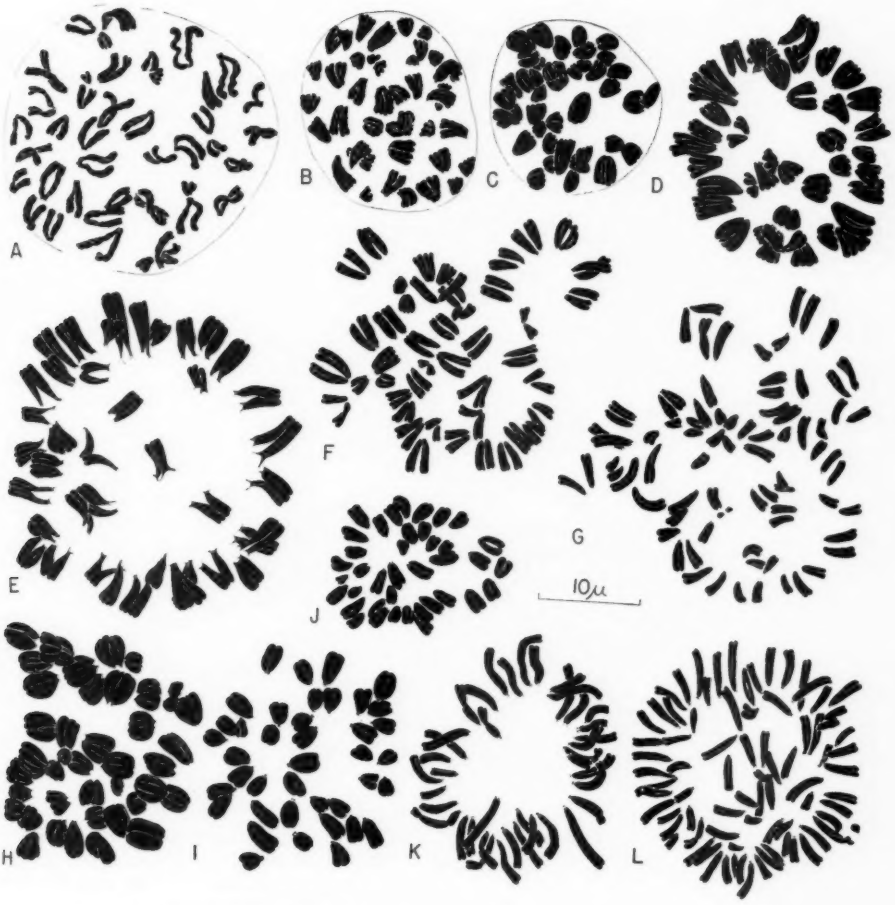
Levan and Hauschka, figure 4

FIGURE 5.—*A–D*: Krebs 2; *E–W*: Sarcoma 37.
A,E: resting nuclei.
B–D: stages of endomitosis.
F–W: separately drawn chromosomes of the endomitotic cycle (*F–L*), and the normal mitotic cycle (*M–W*).
A–E × 1,270; *F–W* × 2,350



Levan and Hauschka, figure 5

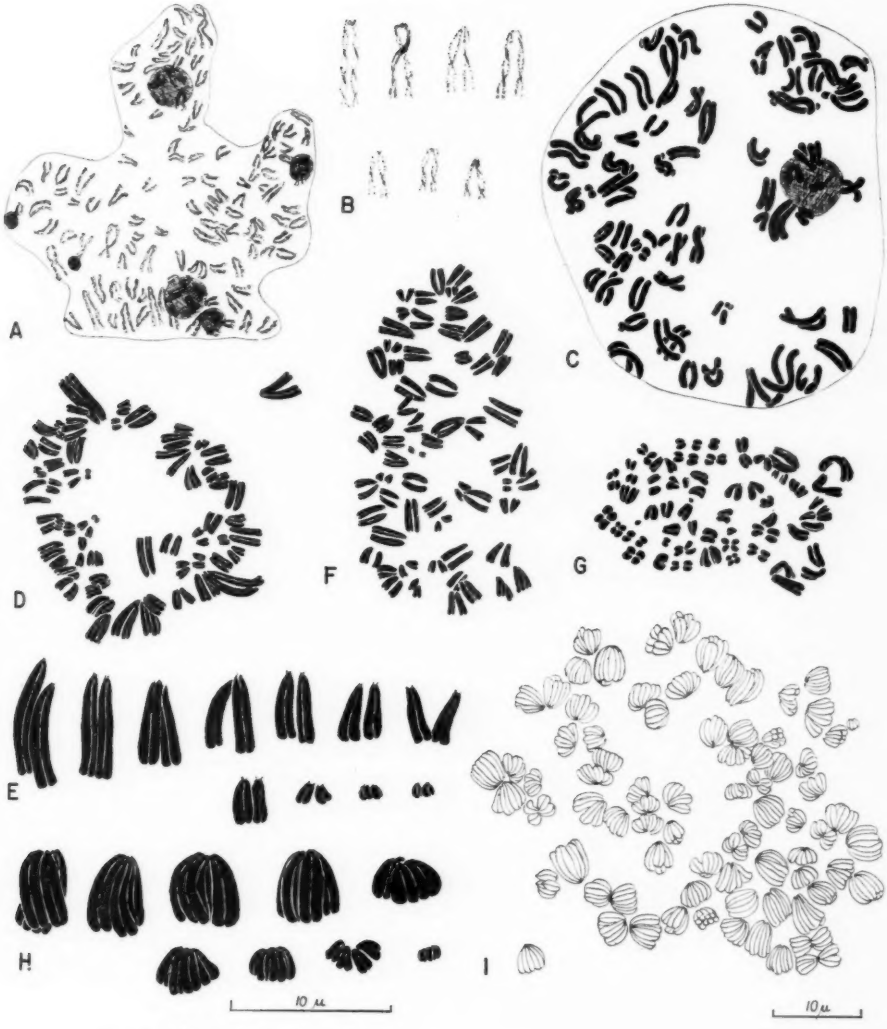
FIGURE 6.—TA3 adenocarcinoma.
A–H: endoreduplication stages.
A–C: prophases.
D–H: metaphases.
I–L: normal mitosis, metaphases
× 1,500



Levan and Hauschka, figure 6

255905—53——3

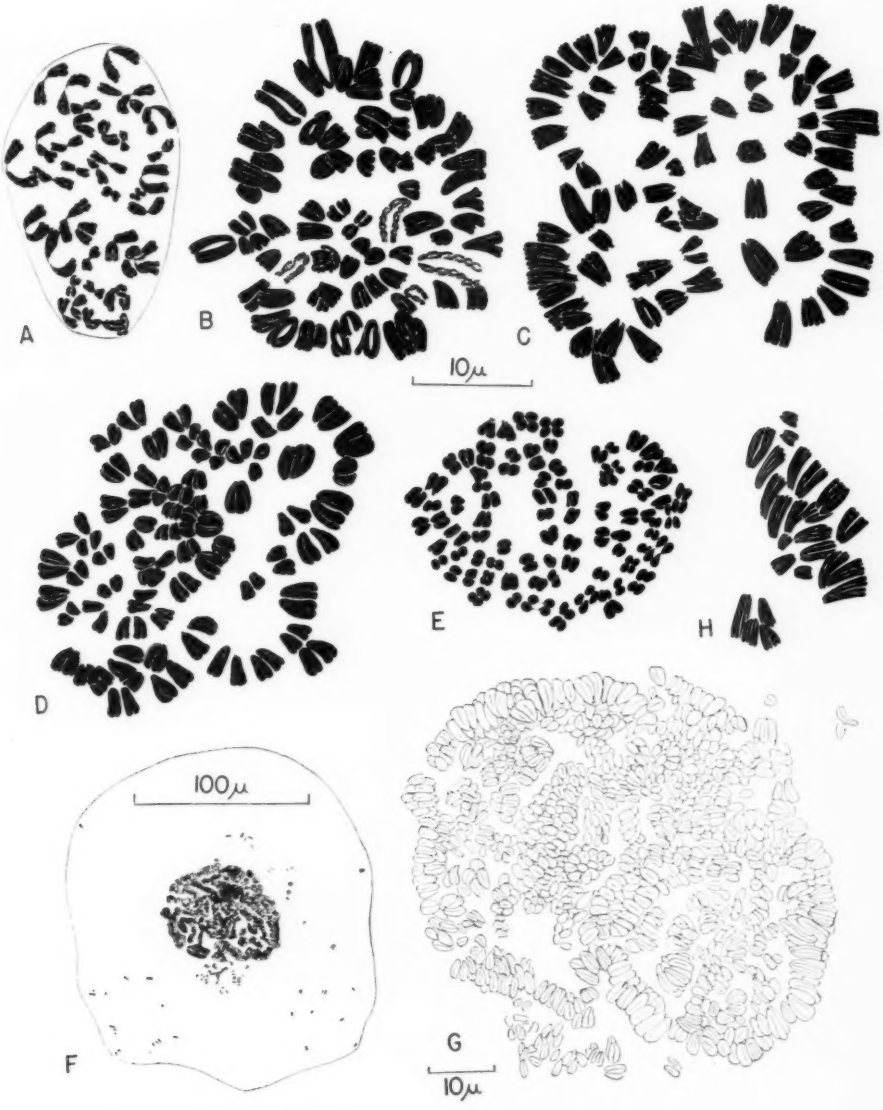
FIGURE 7.—Endoreduplication stages.
A–E: MC1M rhabdomyosarcoma.
F: MC1A rhabdomyosarcoma.
G: Sarcoma 37.
H,I: 15091a carcinoma.
E, H × 2,400; all others × 1,300



Levan and Hauschka, figure 7

FIGURE 8.

A: Krebs 2, endoreduplication prophase.
B: Ehrlich, metaphase of endoreduplication.
C–H: Krebs 2, metaphases of endoreduplication.
F: a giant cell with very high chromosome number.
G: the plate of *F*, roughly represented.
H: detail from *G*.
A–E, *H* × 1,800; *F* × 260; *G* × 970



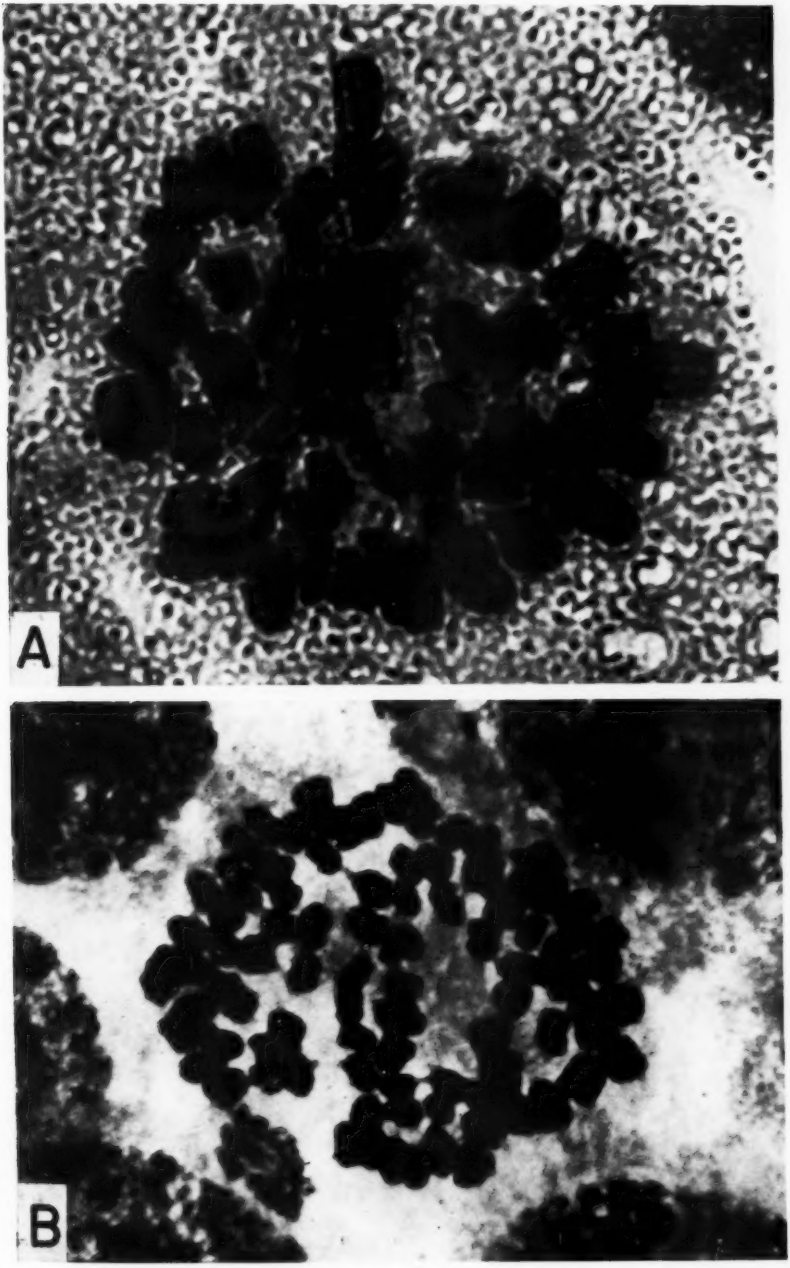
Levan and Hauschka, figure 8

FIGURE 9.—Krebs 2.
A–D: ideogram analyses of two normal mitoses.
E,F: endoreduplication mitosis.
A, *C*, *E* × 900; *B*, *D*, *F* × 1,700

Levan and Hauschka, figure 9

PLATE 1

FIGURE 10.—Photomicrographs of the two endoreduplication metaphases of figures 8*B* and *E*, respectively. *A*: Ehrlich; *B*: Krebs 2. × 3,200



Levan and Hauschka, figure 10

FIGURE 11.—Metaphases with highly increased chromosome number (402 and 522 chromosomes, respectively).
A: Ehrlich tumor. × 1,460
B: Krebs 2 tumor. × 920

Levan and Hauschka, figure 11

255905—53——6

Biological Studies on Stable and Radioactive Rare Earth Compounds. II. The Effect of Lanthanum on Mice Bearing Ehrlich Ascites Tumor [1, 2, 3]

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It is known that rare earth salts combine with nucleic acids *in vitro* to form insoluble complexes (*2*, *3*). Therefore, a study of the biological effects of these elements and their radioactive isotopes *in vivo* seemed of interest. The inhibitory effect of lanthanum upon the growth and metabolism of *Streptococcus faecalis* R has been studied in this laboratory (*4*). The distribution of lanthanum in normal, adult mice was studied with the aid of La^{140} as a tracer (*5*). It was found that the distribution of lanthanum depends upon the route of administration, the dose, and the form (ionized or complex) in which it was administered. Furthermore, it was demonstrated (*5*) that lanthanum, when injected intraperitoneally as $LaCl_3$, remained essentially localized in the peritoneal cavity.

These observations led to an investigation of the effect of stable and radioactive rare earth compounds on rapidly dividing cells, particularly on tumor tissue. The localization of $LaCl_3$ in the abdominal cavity suggested the use of ascites tumors as a suitable object. The test substance may be brought into intimate contact with the actively proliferating tumor cells, thus eliminating variations due to differences in vascularization or secondary effects caused by damage to blood vessels. Ascites tumors have been standardized in several strains of mice and have been extensively used for the assay of chemotherapeutic agents (*cf. 6*, *7*, *8*). The advantages of this type of transplanted tumor are that: the size of the inoculum can be accurately determined; the growth rate is a function of the number of inoculated tumor cells; samples of tumor cells may be withdrawn at various stages for serial cytologic or chemical studies, thus making it possible to ascertain the effects of therapeutic agents on the malignant cells with relative ease (*6*, *7*).

[1] Received for publication February 5, 1953.

[2] A preliminary report (*1*) of these results was presented at the 1952 Annual Meeting of the American Association for Cancer Research.

[3] This work was performed under Contract AT-30-1-880 between Montefiore Hospital and the United States Atomic Energy Commission.

Materials and Methods

Adult strain A and (C57BL × A)F_1 [4] mice weighing 19 to 25 gm. were used in these experiments. The animals were maintained on Rockland mouse pellets, and water *ad libitum*. The original donor used for transplantation of ascites tumor was a strain A mouse that had been inoculated with Ehrlich adenocarcinoma.[5] This tumor had previously been carried through several intraperitoneal transplant passages. Transfers were made every 3 to 5 days into strain A and (C57BL × A)F_1 mice, using as inoculum 0.1 ml. of the milky ascitic fluid containing approximately 12 million tumor cells. All animals thus inoculated developed ascites and gained weight rapidly during the first week. Thereafter the weight gain was less regular and was generally followed by a weight decline preceding death. No spontaneous regressions were noted. At the time of death the ascitic fluid was usually hemorrhagic and contained up to 150 million tumor cells per milliliter. Furthermore, in the majority of animals autopsy revealed the presence of tumor nodules in the mesentery as well as at the site of the inoculation.

For therapeutic experiments, the animals were inoculated with 0.1 ml. of a dilution of ascitic fluid with physiological saline containing the desired number (10,000 to 12,000,000) of tumor cells. Following inoculation the mice were divided into groups comprising 10 to 42 animals. One group served as saline control, another group was treated with radiolanthanum (La^{140}) chloride containing stable lanthanum as a carrier. A third group was injected with stable lanthanum chloride in doses corresponding to the carrier content of the radioactive lanthanum preparation administered to the second group. The doses ranged from 50 to 600 μc, containing 0.5 to 2.5 mg. of the carrier. The treatment consisted of single or repeated injections, starting 1 to 5 days after the inoculation with tumor cells. The preparation of the radioactive $LaCl_3$ solutions and the radiation characteristics of La^{140} have been previously described (*5*). Dosage calculations were based on the values furnished by the Oak Ridge National Laboratory.[6] The criteria used in evaluating the effects of stable lanthanum and radiolanthanum were: changes in body weight, survival time, volume of ascitic fluid at time of death, and findings at autopsy. The mice were weighed frequently during the first week of the experiments (see tables 1 and 2, and text-fig. 3) and the average weight changes in each group were calculated. Mortality data were expressed on a percentage basis. At the time of death the ascitic fluid was aspirated and the volume was measured. In some instances the cell count of the fluid was determined in a hemocytometer. Samples of ascitic fluid were repeatedly obtained for cytologic and cytochemical studies in separate

[4] The strain A mice were obtained from "Stronghold," New Haven, Conn. The (C57BL × A)F_1 mice (the F_1 hybrids of strain C57BL and strain A) were furnished by the Jackson Memorial Laboratory, Bar Harbor, Maine.

[5] This animal was obtained through the courtesy of Dr. T. L. Ambrus, of the Philadelphia College of Pharmacy and Science, Philadelphia, Pa.

[6] The values are only approximate; the problems of standardizing and of accurate dosage determination for La^{140} are under investigation at present.

experiments, since frequent abdominal tappings render the criteria of body weight and survival time less dependable. Autopsies were performed on all animals in order to ascertain whether solid tumors were present in the abdominal cavity. At the time of death the amount of lanthanum present in various tissues and in the ascitic fluid was determined by standard radioactivity measurements (*5*). In the removal of extraperitoneal tissues care was taken to prevent contamination with fluid from the abdominal cavity. The intra-abdominal organs were rinsed with saline, in order to minimize contamination with the radiolanthanum *suspended* in the ascitic fluid. This procedure does not remove La^{140} that is *adsorbed* on organ surfaces, as has been shown by means of radioautographs (*5*). In order to determine whether the ascitic fluid from radiolanthanum-treated mice contained viable tumor cells, fluid was withdrawn several days after lanthanum treatment and injected into the peritoneal cavity of normal mice. Failure to develop ascites and/or solid tumor nodules following the injection indicated the absence of viable cells.

Observations and Results

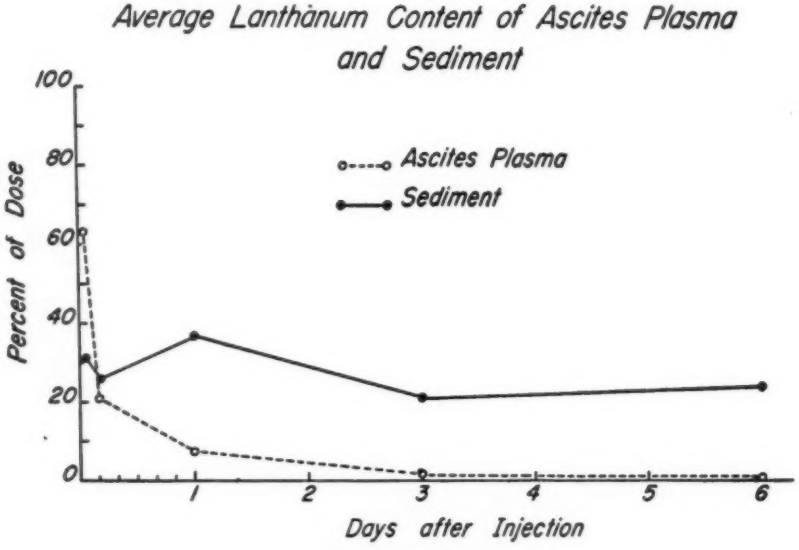
Toxicity.—The toxicity of stable lanthanum chloride, administered *intravenously* to normal mice, has been reported previously (*5*). In order to investigate the toxicity of *intraperitoneally* administered $LaCl_3$, 30 $(C57BL \times A)F_1$ mice were injected with lanthanum chloride containing 4 mg. of La^{+++}. Another 30 mice were used as saline controls. No deaths resulted from acute toxicity. Three months after the injections were given, 28 mice were alive in each group. The animals were sacrificed and autopsied. The only abnormal findings on post-mortem examinations were adhesions and a thickening of the capsule of the liver and the spleen, indicative of an adhesive chemical peritonitis. The highest single dose administered in therapeutic experiments was 0.9 mg., and the highest total dose after repeated injections was 3.2 mg. of La.

Toxicity studies with radioactive lanthanum chloride indicated that mice bearing ascites tumors were apparently more resistant than normal mice, due presumably to the presence of ascitic fluid and ascites tumor cells, which retain a fraction of the administered dose.

Distribution.—Upon intraperitoneal administration of $LaCl_3$ *containing carrier* into normal mice, about 70 percent of the activity could be recovered from the abdominal cavity (*5*). Radioautographs revealed localization of the radioactive lanthanum on the surfaces of the intraperitoneal organs. Studies on the distribution of $LaCl_3$ following intraperitoneal injection into mice bearing ascites tumors essentially confirmed the results obtained with normal mice. Only negligible amounts of La were absorbed from the abdominal cavity.

The distribution of lanthanum in the ascites supernatant and sediment was studied after the intraperitoneal administration of $LaCl_3$ of higher specific activity. No visible precipitate was noted when 0.02 mg. of La was added to ascitic plasma *in vitro*, whereas a precipitate formed after

addition of 0.7 mg. of La per ml. of plasma. On the basis of these observations it seems unlikely that the activity of the sediment is due to precipitable lanthanum-protein particles. Samples were collected 1, 4, 24, 72, and 144 hours after the intraperitoneal injection of radiolanthanum chloride. The amount of the administered dose recovered in the ascites was 94, 47, 44, 22, and 24 percent, respectively. The average volume of the ascitic fluid was 2 ml. at the time the first samples were withdrawn, whereas 6 days later it was 6.7 ml. The distribution of radiolanthanum in the ascitic plasma and the sediment is shown in text-figure 1. The dosage used was 10 μc with 0.02 mg. of La. As can be seen from the figure, the radioactivity in the supernatant declined from its initial value of 63 percent of the dose to less than 1 percent three days later. The activity in the sediment remained high and 6 days after the injection approximately 24 percent of the dose was found in the sediment as compared with 31 percent 1 hour after the injection.

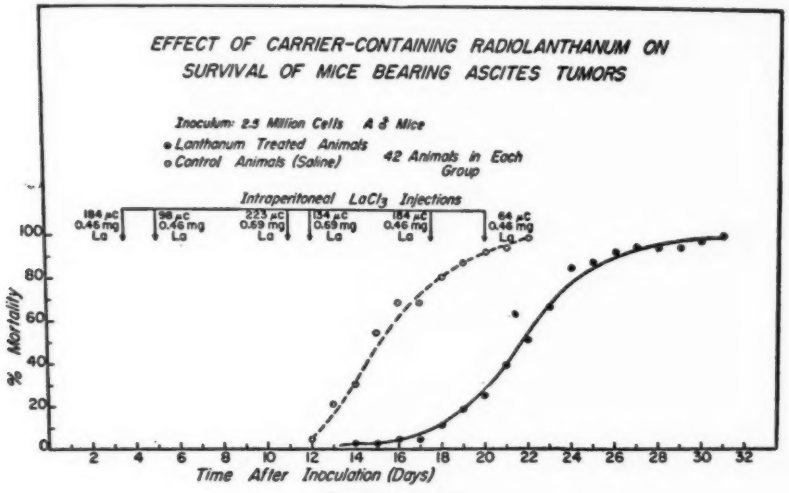


TEXT-FIGURE 1.

Therapeutic experiments.—Approximately 1,000 animals were used in these experiments. Several series of experiments were conducted in which the number of tumor cells in the inoculum ranged from 1 to 12 million. In other series a smaller inoculum of tumor cells (70 to 100 thousand) was used. Representative experiments with the acute as well as the chronic form of the disease are presented herewith.

The effect of radiolanthanum on animals inoculated with 2½ million ascites cells is shown in text-figure 2. Two groups of 42 strain A mice were formed. Treatment with radiolanthanum was started 3 days after inoculation, at which time the control group was injected with saline intra-

peritoneally. In order to maintain the level of radioactivity at approximately 100 to 200 μc for 2 weeks, the injections were repeated as shown in text-figure 2. The survival time of the treated animals was significantly prolonged. On the 16th day following the inoculation the mortality was 70 percent for the control animals and only 5 percent for the lanthanum-treated mice. After 22 days all the control animals had died, whereas 50 percent of the treated mice were still alive. Furthermore, the treated mice gained weight at a markedly slower rate than the control animals (table 1).


EFFECT OF CARRIER-CONTAINING RADIOLANTHANUM ON SURVIVAL OF MICE BEARING ASCITES TUMORS
Inoculum: 2.5 Million Cells A & Mice
Lanthanum Treated Animals
Control Animals (Saline)
42 Animals in Each Group
Intraperitoneal LaCl3 Injections
184 μC 0.46 mg La
98 μC 0.46 mg La
223 μC 0.69 mg La
134 μC 0.69 mg La
184 μC 0.46 mg La
64 μC 0.46 mg La
% Mortality
Time After Inoculation (Days)

TEXT-FIGURE 2.

TABLE 1.—*Changes in average body weight of two groups of mice bearing ascites tumors: radiolanthanum chloride injected intraperitoneally, and saline controls.**

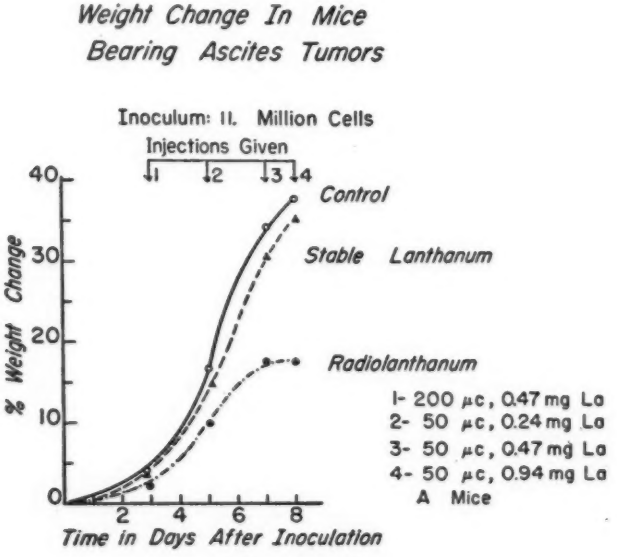
Group	Body weight in grams		
	At time of inoculation	8 days later	Increment
Controls (42 mice)	24.6±0.2†	32.0±0.5‡	7.4
La-treated (42 mice)	25.1±0.3†	27.8±0.4‡	2.7

*For size of inoculum and treatment see text-figure 2.
†The significance ratio of the difference of the means of the two groups is 1.4.
‡The significance ratio of the difference of the means of the two groups is 6.7.

The control mice gained an average of 7.4 grams in 8 days. The treated animals, on the other hand, had gained only 2.7 grams during the same period.

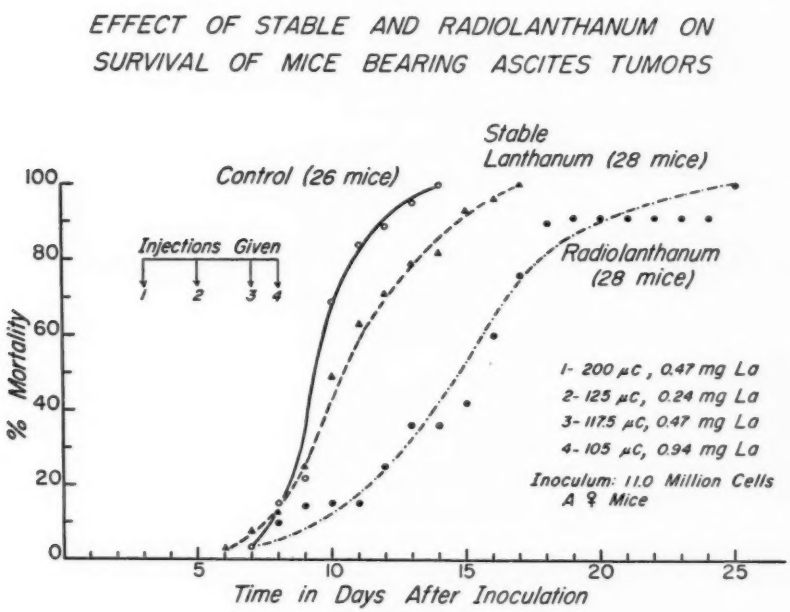
Since, as can be seen from text-figure 2, the amount of carrier lanthanum injected was relatively high, the experiment was repeated to test the effect

of the stable lanthanum itself. In this experiment, 3 groups of strain A mice were inoculated with approximately 11 million cells. This inoculum was almost 5 times as large as in the experiment previously discussed and the survival time of the control mice was correspondingly shorter. Treatment was started 3 days after inoculation. One group of 26 mice (group A) served as saline controls, while groups B and C, comprising 28 animals each, were treated with radioactive lanthanum containing carrier, and stable lanthanum, respectively. The animals in group B received 4 injections. The first injection contained approximately 200 μc, the subsequent injections 50 μc each; the total carrier content was about 2.1 mg. of La^{+++}. Equivalent amounts of stable lanthanum were administered to animals of group C. The changes in body weight and the mortality data shown in text-figures 3 and 4 indicate that there was no significant effect of stable lanthanum, whereas radiolanthanum produced marked changes when compared with the saline-treated control group. On the 8th day after inoculation the control mice showed a gain of approximately 40 percent of their original body weight, whereas the radiolanthanum-treated animals had gained less than 20 percent (text-fig. 3). The weight



TEXT-FIGURE 3.

change in the stable lanthanum group did not differ significantly from that of the controls. Text-figure 4 shows that on the 11th day after inoculation the mortality of the saline group (A) was 85 percent, whereas only 18 percent of the animals had died in the radiolanthanum-treated group (B). The stable lanthanum (group C) had a slight, but probably insignificant effect.

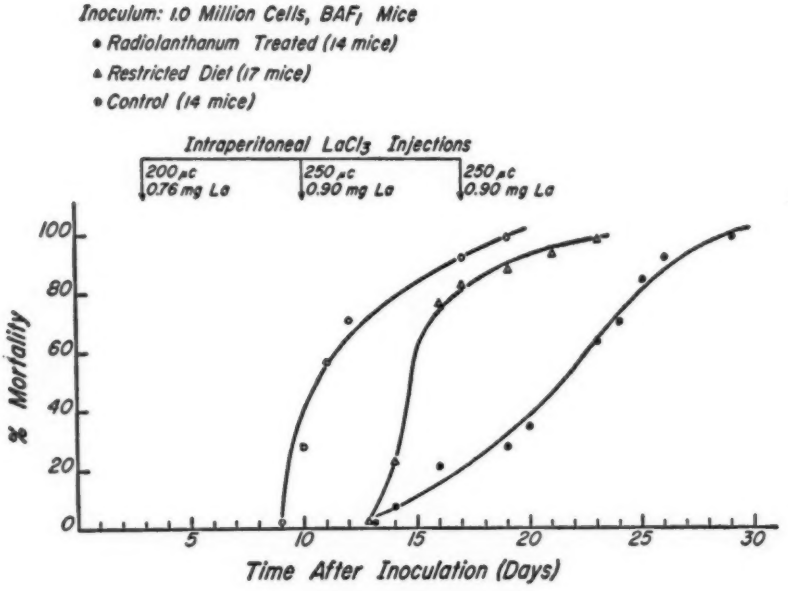


TEXT-FIGURE 4.

In order to determine whether the effect of radiolanthanum on ascites tumor may be due to a direct inhibition of growth or to malnutrition resulting from radiation sickness, the following experiment was performed. (C57BL × A)F_1 mice were inoculated with 1 million tumor cells and divided into three groups (see table 2 and text-fig. 5). The mice in groups A and C were offered Rockland mouse pellets and water *ad libitum* and the daily food intake was determined. The food consumption of the animals in control group A declined on the 7th and 8th days after inoculation to approximately two-thirds, and from the 9th day on to about 15 percent, of their previous dietary intake. This decrease in food intake coincided with the decline in body weight and the sharp increase in mortality. The amount of food consumed by the radiolanthanum-injected animals (group C) decreased to one-third on the day after treatment but returned to the pretreatment level within a few days. A temporary drop was also noted on the days of the second and third radiolanthanum injection, with prompt return to normal intake on the subsequent days. In contrast to the animals in groups A and C, mice in group B were kept on a restricted diet. Five days after inoculation the food intake was restricted to one-third of the amount previously consumed. This dietary restriction was maintained throughout the entire experiment. The data in table 2 and text-figure 5 indicate that at the time of death the animals in group B had approximately as much ascites as the control mice fed *ad libitum* (group A) and that their survival time, although somewhat increased, was considerably less than that of the radio-

lanthanum-treated mice (group C). Since the dietary restriction in group B was more severe and more prolonged than the temporary decline in food intake of the radiolanthanum-treated animals, it may be assumed that the decrease in the formation of ascites and the increased survival time of the latter group were mainly caused by radiolanthanum.

EFFECT OF RESTRICTED DIET AND RADIOLANTHANUM ON SURVIVAL OF MICE BEARING ASCITES TUMORS



TEXT-FIGURE 5.

It should be mentioned that, upon autopsy, the radiolanthanum-treated animals showed solid intraperitoneal tumor nodules. Klein (7) has shown that inoculation with *small* numbers of ascites cells produces no ascites, but *solid tumors* appear after a relatively *long latent period.* In contrast, the appearance of a typical ascites tumor, that forms rapidly after inoculation and causes death of the animal in a short period of time without developing a solid tumor, depends upon the initial presence of a large number of tumor cells. Thus our observation may be regarded as further evidence that the multiplication of a large proportion of the tumor cells originally present was inhibited by the radiolanthanum treatment. The surviving tumor cells behaved like a small inoculum,

seeding in the mesentery to form solid nodules. These nodules may not be as readily accessible to the radiolanthanum as the free cells in the ascitic fluid.

TABLE 2.—*Changes in average body weight and ascites volume of 3 groups of mice bearing ascites tumors: controls, restricted diet, and radiolanthanum-treated**

Time	Body weight in grams		
Days after inoculation	Group A Controls 14 mice	Group B Restricted diet 17 mice	Group C Radiolanthanum-treated 14 mice
0	21.2	21.3	20.9
3	21.8	21.7	22.1
5	23.4	23.2	19.9
6	24.0	22.3	20.1
9	30.0	22.3	20.4
11	27.3	24.8	21.8
14	28.6	25.4	23.0
Average increment in 15 days	7.4	4.1	2.1
	Volume of ascites (*ml.*)		
At time of death	5.4 (3.5–8.0)†	5.0 (1.0–9.0)	2.7 (0–6)

*For size of inoculum and treatment see text-figure 5.
†The figures in parenthesis indicate the range of the volume of ascites.

In all the experiments so far described, large numbers of tumor cells were inoculated. As was mentioned previously, the course of the disease is influenced by the size of inoculum. It was therefore decided to study the therapeutic effect of lanthanum on more slowly growing ascites tumors. Three groups, of 24 to 25 (C57BL $\times$ A)F_1 mice each, were formed. Approximately 75,000 cells were inoculated and the treatment started only 5 days after inoculation. The dose of radiolanthanum chloride administered had an activity of approximately 250 μc.

Twenty-three of the 25 radiolanthanum-treated animals were still alive on the 25th day after inoculation (table 3), but only 12 of the saline control group survived. On the 45th day most of the control mice had died, whereas 17 radiolanthanum-treated mice were still alive. On the 102nd day 8 radiolanthanum-treated animals were alive, as compared with 2 animals of the control group. One year after the beginning of the experiment all these surviving mice were sacrificed and autopsies performed. No evidence of tumor growth was found in any of these animals. The consistent differences between the two groups observed throughout the entire experiment would seem to suggest that the survival of some of the 8 mice in the experimental group may be attributed to radiolanthanum treatment.

TABLE 3.—*Survival data for groups of mice bearing ascites tumors, treated with stable lanthanum, radioactive $LaCl_3$, or saline**

Treatment	No. of mice per group	No. of animals alive			
		25 days†	38 days	45 days	102 days
Control	25	12	7	3	2
Radiolanthanum	25	23	20	17	8
Stable La	24	15	12	9	3

*Number of tumor cells inoculated: 75,000. The dose of radioactivity was approximately 250 μc; the dose of La^{+++} was 0.9 mg. Treatment was started 5 days after inoculation.
†Time in days after inoculation.

The effect of a single large dose of radiolanthanum (400 μc) was studied in the next experiment (table 4). About 45,000 tumor cells were inoculated into 40 strain A mice. Twenty animals received approximately 400 μc of La^{140} as the chloride in one injection 3 days after the inoculation; 20 mice served as saline controls. Ten of the treated animals died a few days after the injection, presumably due to radiation. Five mice were sacrificed from both the control and the radiolanthanum-treated groups 7 days after the radiolanthanum injection. The ascitic fluid was aspirated and measured. At this early stage of the disease no significant differences were found between the treated and control animals. Twelve days after the inoculation, another 5 mice were sacrificed in each group. At this time major differences between the two groups were found, as shown in table 4. A small amount of fluid was present in only one of the treated animals, whereas all five controls had ascites. Radiolanthanum given in this dose almost completely suppressed ascites formation and destroyed the tumor cells. Viability studies by the subculturing of ascitic fluid samples showed that the fluid obtained from the control mice induced ascites tumor formation at the usual rate, whereas neither ascites nor solid tumors developed when the fluid obtained from the *one* radiolanthanum-treated animal in which it was found was injected into normal mice.

TABLE 4.—*Weight changes and ascites volume in groups of mice bearing ascites tumors, treated with radiolanthanum chloride or with saline**

Mouse No.	Weight change		Ascites	
	Controls (*gm.*)	Treated animals (*gm.*)	Controls (*ml.*)	Treated animals (*ml.*)
1	+7.1	−2.4	4.7	0
2	+6.4	−1.0	8.0	0
3	+6.1	−7.3	8.0	0
4	+6.4	−4.9	8.5	1
5	+8.0	+0.3	7.5	0

*A dose of 400 μc was injected 3 days after inoculation of ascites, and the animals were sacrificed 9 days thereafter.

Discussion

The reasons for the selection of ascites tumors for the study of the effect of radiolanthanum on malignant cells have been briefly outlined. The observations here reported show that the lanthanum remains largely in the abdominal cavity following intraperitoneal administration of $LaCl_3$. Furthermore, radioassays of the sediment of the ascites (see text-fig. 1) show that it contains a large proportion of the radiolanthanum administered. Initially, the ascitic plasma had approximately twice as much activity as the sediment, whereas six days later only traces of activity were evident. The close contact of radiolanthanum with the tumor cells is favorable for therapy and is consistent with the marked inhibitory effect of this radioisotope upon the ascites tumor. The criteria used to measure the inhibitory action of lanthanum were survival time, changes in body weight as indicators of ascites accumulation, ascites volume, and number of tumor cells. The degree of inhibition depends upon the relation of the dose of radiolanthanum to the number of tumor cells. Our observations indicate that the therapeutic effect of carrier-containing $La^{140}Cl_3$ is, in all probability, due to the radioisotope rather than the stable lanthanum La^{139}, since control experiments with La^{139} gave essentially negative results. On the other hand, *in vitro* studies (*9*) showed that relatively high concentrations of stable lanthanum ions produced significant effects on the respiration of ascites tumor cells in the presence of glucose.

Although the lanthanum was administered in ionized form as the chloride, it is possible and indeed probable that the rare earth ions interacted with components of the ascitic fluid to form colloidal lanthanum-protein complexes similar to those produced by the introduction of $LaCl_3$ into the blood stream (*5*). Titration and electrophoresis experiments performed in this laboratory indicate that lanthanum ions react with the proteins present in ascitic plasma to form soluble and insoluble compounds.

The differences in distribution observed upon administration of carrier-containing (*5*) and carrier-free lanthanum (*10*) are probably attributable to the fact that infinitesimal amounts of La^{140} show a chemical behavior different from that of the weighable quantities of La^{139} plus La^{140} employed in the present experiments. In this connection, reference is made to the observation of J. Lewin (*11*) that one mole of $LaCl_3$ forms a *soluble* complex with one mole of human mercaptalbumin. Furthermore, the experiments of Dobson *et al.* (*12*) have demonstrated a variation in distribution of colloidal complexes of zirconium, yttrium, and niobium as a function of particle size.

In this connection it might be mentioned that a relatively large number of rare earth isotopes of widely differing half-life and radiation characteristics, but with closely similar chemical reactivity, have recently become accessible in the form of fission products or through neutron bombardment of stable isotopes (*13*). The possibility of selecting a radioisotope with the most favorable radiation spectrum and physical and biological half-life in relation to the radiation susceptibility of a specific type of tumor cell

offers a promising therapeutic approach. Experiments with yttrium[90] are in progress.

Summary

1) The effect of intraperitoneally administered radiolanthanum chloride was studied in mice bearing Ehrlich ascites tumor.

2) Lanthanum was found concentrated in the sediment of ascitic fluid containing tumor cells, whereas the initial high lanthanum content of the supernatant fluid declined rapidly to trace levels.

3) Marked inhibitory effects of radiolanthanum were noted, as judged by increased survival, decreased ascites formation, and decreased number of viable tumor cells. The relationship between the degree of inhibition, dose of radiolanthanum, and size of inoculum was illustrated. Stable lanthanum under comparable conditions had a minimal inhibitory effect.

The authors wish to thank Mr. Bruno Shrenger for valuable technical assistance.

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Nutritional Deficiency Produced in the Mouse by Feeding Bentonite [1,2]

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When mice were fed semisynthetic diets to which large amounts of bulk-forming material had been added, animals receiving a diet of 50 percent bentonite grew very slowly, developed fatty livers, and, after some months, eleven of twelve mice developed hepatomas [Wilson (*1*)]. Mice on similar diets with Ruffex or barium sulfate added showed good, if not normal, growth and their livers were normal. The similarity of the response of the bentonite-fed animals to that of mice in other experiments, fed on low-protein diets deficient in choline (*2*), suggested that these mice might also be subject to choline deficiency, possibly due to the fact that bentonite is a base-exchange silicate and thus removes choline from the contents of the intestine. The experiments herein discussed were undertaken to test this possibility.

Materials and Methods

The mice used were all of inbred strains BUA, BUB, or BUC, maintained in air-conditioned rooms at about 25° C. and fed routinely on Purina laboratory chow. The basal diet in these experiments is as follows: casein (Borden's "Edible") 20%, cornstarch (Argo) 40%, Crisco 20%, corn oil (Mazola) 4%, salt mixture (GBI-USP No. 2) 6%, yeast (GBI) 8%, and cod-liver oil (Squibb) 2%. This entire diet is mixed with the bulk-former in the required percentage by weight or, in some experiments, the bulk-former may replace a part of the cornstarch or Crisco. The bentonite was supplied by the American Colloid Company as "Volclay Bentonite." The montmorillonite was supplied by the Milwhite Company, Inc. The Ruffex is a pure cellulose material obtained from the Fisher Scientific Company, and the barium sulfate ("Baridol," a product especially prepared for gastroenterological roentgenography) was supplied by the Pacific Chemical Laboratories.[4]

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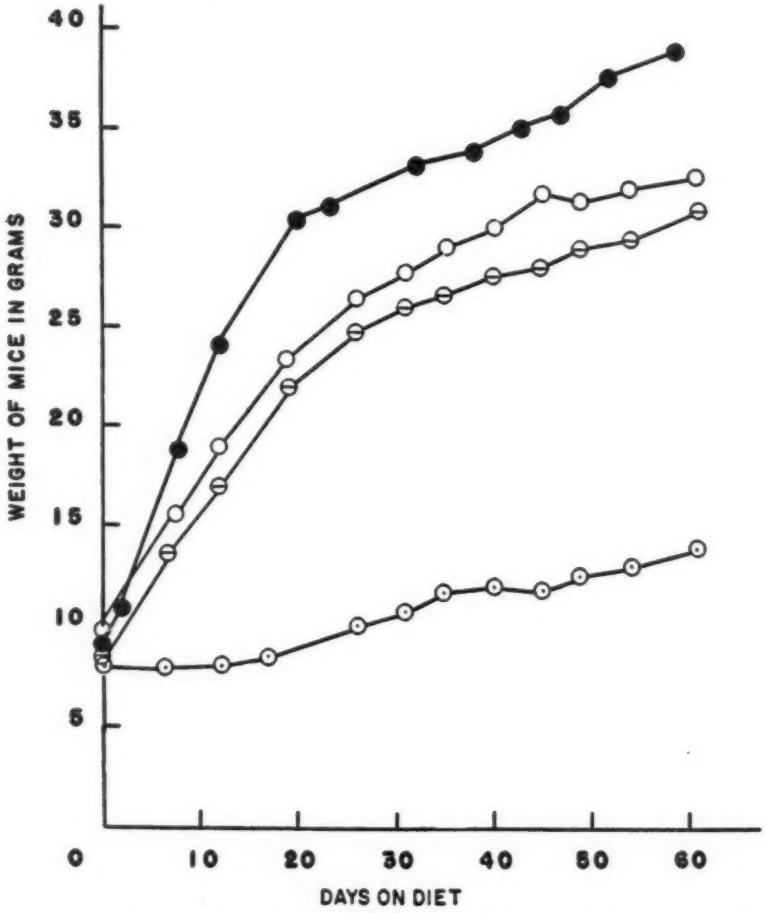
[3] With the technical assistance of Miss Mary Day, Mrs. Harriet Parkhurst, and Miss Carolyn Swaney.

[4] Thanks are due to the following for generous supplies of materials: American Colloid Company, Chicago, Ill.; The Milwhite Company, Inc., Houston, Tex.; Pacific Chemical Laboratories, San Francisco, Calif.; and Merck & Co., Inc., Rahway, N. J.

All mice were weighed at least twice a week. At sacrifice, pieces of liver were fixed in Bouin's fluid, sectioned at 5 μ and stained with Delafield's hematoxylin and eosin. The liver was also fixed in 10-percent formalin and stained for fat with Sudan IV.

Experimental Results

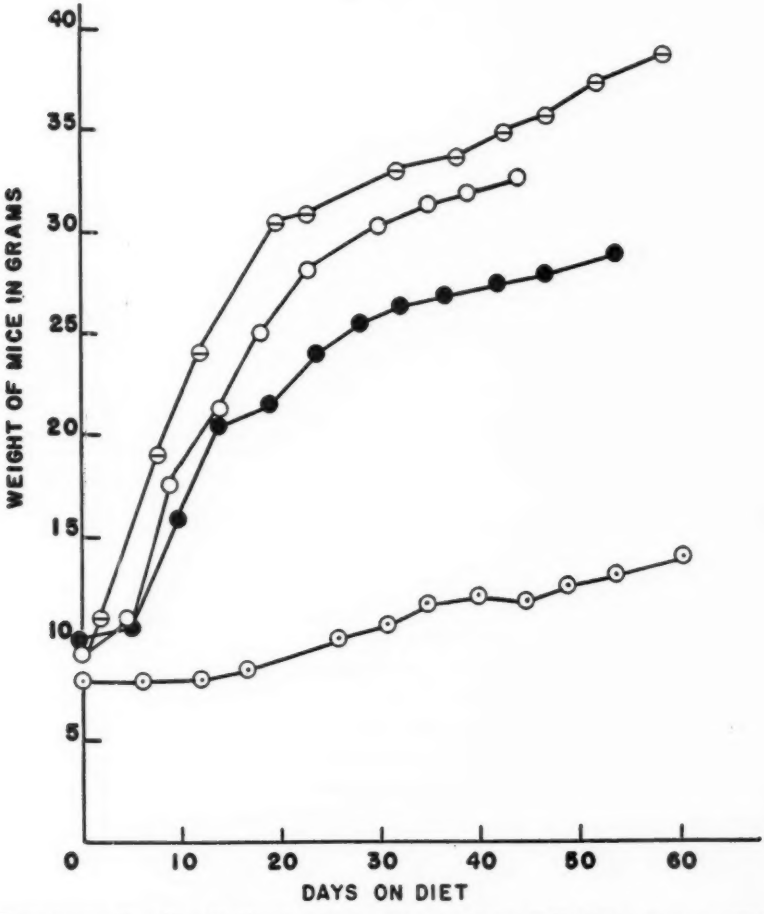
Two male and two female 18-day-old mice were placed on the basal diet and on diets with 10%, 25%, or 50% bentonite added to the basal diet (text-fig. 1) and maintained on these diets for 61 days. Those on the 10% and the 25% bentonite grew at a rate slightly below that of the controls,



TEXT-FIGURE 1.—Growth of male mice on the basal diet mixed with bentonite. Each curve represents the average weights of two mice placed on the diet at 18 days of age. ●—100% basal diet; ○—10/90 bentonite-basal diet; ⊖—25/75 bentonite-basal diet; ⊙—50/50 bentonite-basal diet.

but those on the 50% bentonite grew very slowly. The histologic sections of the livers of these retarded animals showed an abundance of fat vacuoles, whereas the livers of the other mice were normal.

When 50% Ruffex or 50% barium sulfate was used in place of the 50% bentonite in similar experiments (text-fig. 2), nearly normal increase in weight occurred, but when 50% montmorillonite (another clay related to bentonite) was used, the growth was extremely retarded. This indicates that the effect of the clay is not due to bulk alone and that it is not due to a contaminant in the bentonite unless the same contaminant occurs in a similar clay from a different source. The slightly lower rate of growth



TEXT-FIGURE 2.—Growth of male mice on diets containing 50% Ruffex, barium sulfate, or bentonite. Each curve represents the average weights of two mice placed on the diet at 18 days of age. ○—100% basal diet; ⊙—50/50 bentonite-basal diet; ●—50/50 Ruffex-basal diet; ⊖—50/50 barium sulfate-basal diet.

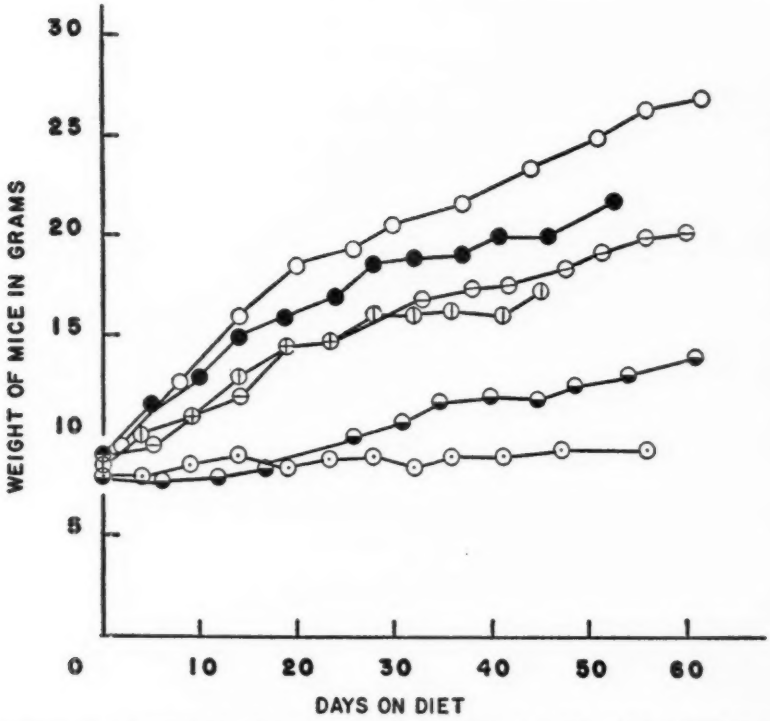
on Ruffex compared with barium sulfate may be attributed to the greater bulk of the former, which may result in inanition that may not be otherwise detectable.

The possibility that the extreme retardation on the 50-percent bentonite diet might be due to partial inanition was studied in an experiment in which for 16 days the food intake of two young adult (70-day-old) mice on each of the following diets was studied: basal, 50/50 bentonite-basal, and 50/50 Ruffex-basal. During this period the animals on the basal diet gained slightly in weight, those receiving 50 percent Ruffex held their weight, and those receiving 50 percent bentonite lost weight slightly. The average daily food intake per mouse for each group was as follows: basal, 3.37 gm.; 50% Ruffex, 5.92 gm.; and 50% bentonite, 6.14 gm. Since half the intake of the last two groups is inert material, the actual food intake was: basal, 3.37 gm.; 50% Ruffex, 2.96 gm.; and 50% bentonite, 3.07 gm. While the somewhat decreased food intake might account for the fact that the rate of growth on the 50-percent Ruffex diet is slightly less than on the basal diet, it does not explain the extreme retardation on the 50-percent bentonite diet.

A series of experiments was performed to counteract the retarding effect of the 50/50 bentonite-basal diet (text-fig. 3). When the bentonite was included in the diet, replacing 10%, 20%, or 30% of the cornstarch, all mice grew steadily at a nearly normal rate and their livers were normal. When 50% bentonite replaced 35% cornstarch and 15% Crisco, growth was moderately retarded, but not as completely so as on the 50/50 bentonite-basal diet, and the livers were normal. When the casein was doubled (vitamin-test casein was used so that unknown factors in the "edible casein" were not increased), or when the yeast, cod-liver oil, and salts were doubled at the expense of cornstarch, moderate growth occurred and the livers of the mice were normal. It would therefore appear that the retardation of growth and the fatty livers on the 50/50 bentonite-basal diet were not due to the bulk of the bentonite and consequent inanition, but to some nutritional deficiency.

The fatty livers, in addition to the retardation of growth on the 50/50 bentonite-basal diet, suggested a choline deficiency. When 0.15% choline chloride was added to the basal diet (text-fig. 3), moderate but not normal growth occurred and the livers were normal. On the other hand, when *d,l*-methionine, which should have a lipotropic action, was added at a level of 0.25% or 0.5% to the basal diet, the slight growth that occurred with the 50/50 bentonite-basal diet was suppressed, and at the 0.5% level none of the mice survived the 60-day test period. The methionine did not prevent fatty livers. Thus, while added choline and added casein correct to some extent the harmful effects of the 50/50 bentonite-basal diet, added methionine does not.

The possibility that a deficiency in choline is involved in the extreme retardation of growth suggested the use of a small amount of bentonite in the diet—low in protein and deficient in choline—used in our previous experiments on choline deficiency in the mouse [Wilson and Leduc (*3*)].



TEXT-FIGURE 3.—Growth of male mice on 50% bentonite with modified basal diets. Each curve represents the average weight of two mice placed on the diet at 18 days of age.

○—50% bentonite replacing 35% cornstarch and 15% Crisco in the basal diet.
●—50/50 bentonite-basal with doubled casein replacing half the cornstarch.
◐—50/50 bentonite-basal with doubled yeast, cod-liver oil, and salts replacing part of the cornstarch.
⊖—50/50 bentonite-basal with added 1.5 gm. choline chloride in 1,000 gm.
⊙—50/50 bentonite-basal with added 5 gm. *d,l*-methionine in 1,000 gm.
◒—50/50 bentonite-basal diet.

When placed on the low-protein, choline-deficient diet, weanling mice grew very slowly but frequently survived for many months and eventually developed hepatomas. When coramine, which is detoxified by transmethylation, was added to this diet the severity of the deficiency was increased, the weanling mice failed to grow, and survived for only short periods (*3*). When a diet of 10% bentonite and 90% of the low-protein, choline-deficient diet was used, the slight growth was also eliminated. The mice maintained their weight but did not thrive. The first one died on the 35th day and the last one was sacrificed on the 88th day because it seemed on the verge of death. The livers of all the mice were extremely fatty. It would seem, then, that even a small amount of bentonite, when added to a diet already deficient in choline, enhances the deficiency.

Discussion

Bentonite, far from being the inert material we had expected it to be, turned out to be a very active one. This activity is apparently due to its properties as a base-exchange silicate. Freundlich [(*4*) p. 34] states that bentonite may be considered to be a colloidal electrolyte with a very large micronic anionic micelle and small exchangeable cations. Because of the importance of this property in soil chemistry, it has been extensively studied by Grim (*5*) and Ross and Hendricks (*6*). Permutit, a synthetic base-exchange mineral, takes up choline and from it the choline is readily eluted [Whitehorn (*7*)]. Organic as well as inorganic cations may be involved in the base-exchange activity of bentonite and the larger ones are less easily removed, apparently because they are held by Van der Waals forces in addition to the Coulomb forces of the simple exchange [Hendricks (*8*)]. It is then possible that by its base-exchange capacity, bentonite may remove choline from the intestinal content stoichiometrically and hold it so that it is removed from the body in the feces. Whether or not this would occur in such a complex situation as the intestine could not, perhaps, be predicted because so many factors are involved, and furthermore it seems possible that other cationic materials might also be removed.

The experiments reported here would indicate that when substantial amounts of bentonite are included in the diet of the mouse, choline is removed and this determines the principal characteristics of the deficiency. There may be a slight tendency toward inanition when 50 percent of the diet is not food. When Ruffex or barium sulfate are used, growth is, at the most, only slightly inhibited and no other signs of deficiency appear. The occurrence of fatty livers in mice fed the 50/50 bentonite-basal diet, and not with the same diet when choline or protein are added, indicates choline deficiency. The addition of 10 percent of bentonite to the basal diet produces little or no effect, but when added to a diet low in protein and already deficient in choline, the slow growth that this diet permits is abolished. It would be difficult to explain these phenomena otherwise than by the removal of choline.

On the other hand, it is possible that some additional factor is involved in the deficiency. This seems clearly indicated by the failure of the added choline to restore growth completely to its normal level. It is also indicated by the complete failure of added methionine to produce growth or even to insure maintenance of body weight, for methionine should serve as a source for the labile methyl groups involved in choline deficiency. It is possible, for example, that pyridoxine, which is also a base, may be removed and that the large amount of added methionine enhances this deficiency to the point where it becomes a serious one. Another possibility is that removal of diamino acids by the bentonite produces an imbalance of amino acids which becomes a serious matter when methionine is added to the diet. Further experiments to test such possibilities are in progress.

Summary and Conclusions

Mice fed on a 50/50 bentonite-basal semisynthetic diet fail to grow and develop signs of choline deficiency, including fatty livers. This does not occur in animals fed a diet composed of 50 percent of either Ruffex or barium sulfate and 50 percent of the basal diet.

Growth on the 50-percent bentonite diet is only partially restored when choline or additional protein are added to the diet, but the fatty livers do not appear. Methionine has no preventive or corrective effect.

It is concluded that the bentonite, as a cation-exchange silicate, removes choline from the intestinal contents thus producing the deficiency, but that in all probability other materials may be removed so that the deficiency is not a simple one.

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Hepatomas in Mice on a Diet Containing Bentonite [1,2]

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Mice fed an adequate semisynthetic diet mixed with an equal weight of bentonite failed to grow normally and developed fatty livers. Further experiments indicated that this was due to a deficiency of choline and possibly of other factors, removed from the intestinal contents by bentonite due to its base-exchange capacity (*1*). Such mice maintained for long periods developed hepatomas. This is consistent with the experience of Engel *et al.* (*2*) with rats on choline-deficient diets and with our own experience with mice on diets low in protein and deficient in choline (*3*). It is the purpose of this paper to discuss the occurrence and development of these hepatomas.

Materials and Methods

The mice used were strains BUA, BUB, and BUC, maintained in this laboratory for use in a program of investigation of the histophysiology and histopathology of the mouse liver. They have been inbred for over twenty generations and selected for excellence of growth and reproduction. They are maintained in an air-conditioned room at about 25° C. and routinely fed Purina laboratory chow.

The basal diet used is as follows: casein (Borden's "Edible") 20%, cornstarch (Argo) 40%, Crisco 20%, corn oil (Mazola) 4%, salt mixture (GBI-USP No. 2) 6%, yeast (GBI) 8%, and cod-liver oil (Squibb) 2%. The bentonite is obtained from the American Colloid Co. as "Volclay Bentonite." The low-protein, choline-deficient diet is a modification of that of Lowry *et al.* (*4*) made up as follows: casein ("Vitamin Test" GBI) 4%, cornstarch (Argo) 86.5%, cod-liver oil (Squibb) 2%, Wesson oil 3%, cystine 0.5%, and vitamins as follows (per 1,000 gm. of diet): pyridoxine 5 mg., thiamine 5 mg., niacin 10 mg., Ca pantothenate 50 mg., riboflavin 10 mg. Mice on these diets are kept in cages with wire mesh bottoms and weighed at least once a week.

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[2] Supported in part by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and from the Rhode Island Cancer Society.
[3] With the technical assistance of Miss Mary Day, Mrs. Harriet Parkhurst, and Miss Carolyn Swaney.

In order to follow changes in the liver, some of the mice received intraveneous injections of thorotrast before the experiment was begun (*5*). Young mice, 35 days of age, received three injections of 0.1 cc. of thorotrast intravenously, given on alternate days. X-ray pictures were taken at intervals, usually once a month.

At sacrifice, pieces of the liver were fixed in Bouin's fluid, sectioned at 5 μ, and stained in Delafield's hematoxylin and eosin. In some cases pieces of liver were also fixed in Rossman's fluid and stained for glycogen by the Bauer-Feulgen method. The liver was also fixed in 10-percent formalin and stained for fat with Sudan IV and for reticular fibers by Gomori's method.

Experimental Results

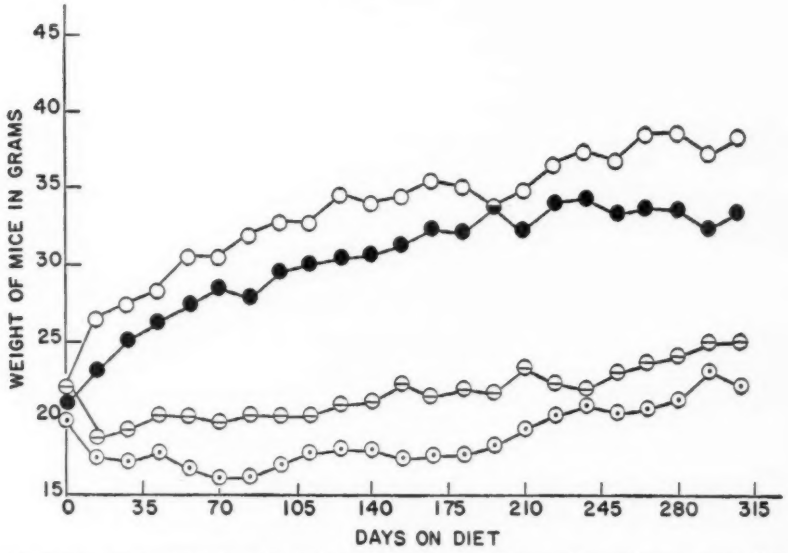
In all, 12 mice have been maintained on the 50/50 bentonite-basal diet for periods long enough to develop hepatomas (over 200 days), and hepatomas were found in all but one. Eight of these had had thorotrast injections and X-ray pictures had been taken. Figures 1 and 2, taken on the 167th and 200th day on the diet, show the development of one hepatoma—pictured at sacrifice on the 203rd day in figure 3. The length of treatment and incidence of tumors are given in table 1; the growth curves for one experiment are given in text-figure 1.

TABLE 1.—*Tumor incidence in mice fed the various diets, and controls*

Experimental treatment	Strain	No.	Period of treatment (*days*)	No. with tumors
50/50 Bentonite-basal diet	BUC	10	240–480	9
50/50 Bentonite-basal diet	BUB	2	203–269	2
Controls, basal diet, 51 A	BUC	7	211–375	1
	BUB	2	283–294	0
50/50 Ruffex-basal diet	BUC	9	280+	0
Thorotrast injections	BUC (varied)	18	216–571	0
Low-protein, choline-deficient diet	BUC	7	232–437	2
	BUA	2	444	1
BUC mice from stock	BUC	24	484–575	0

The occurrence of a hepatoma in one control animal that had been fed on the basal diet, and had been injected with thorotrast, necessitated a survey of strain BUC mice for spontaneous hepatomas. In 24 mice taken from stock, ranging from 484 to 575 days of age, there were no hepatomas. Since all our strains of mice have been developed for and extensively used in liver studies, we have been on the lookout for spontaneous hepatomas and until the present experiments have found only one in animals not on diets planned for the production of hepatomas;

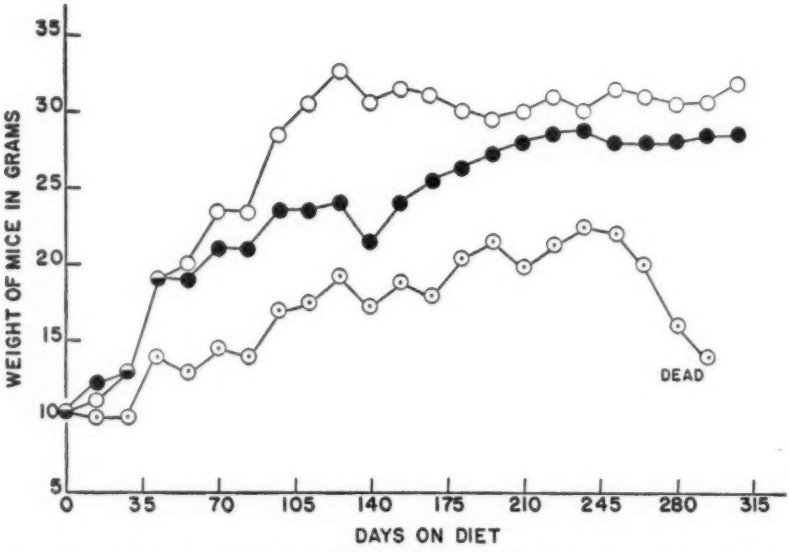
the one case was in strain BUA. In the present experiments only one hepatoma has been found in nine control mice. The alternate possibility, that the hepatoma in this one control might have been caused by the injected thorotrast, was also considered because of the fact that the thorium dioxide is radioactive. However, in 18 animals (not in these experiments) that had lived for 216 to 511 days after the injection of thorotrast, no hepatomas have been found.



TEXT-FIGURE 1.—Weights of strain BUC mice as follows: ○—male on basal diet; ●—female on basal diet; ⊖—Average of three males on 50/50 bentonite-basal diet; ⊙—average of three females on 50/50 bentonite-basal diet. All mice had received 3 injections of 0.1 cc. of thorotrast intravenously and were placed on the diets at 35 days of age. All mice on the 50/50 bentonite-basal diet, with the exception of one male, developed hepatomas.

The production of hepatomas in mice by feeding a diet low in protein and deficient in choline has already been reported (*3*). These experiments were conducted primarily to study the histologic and cytologic changes during the development of cirrhosis. Animals were placed on the diet at weaning, were sacrificed regularly, and only a few lived to the period when hepatomas might be expected. Furthermore, the diet used—a modification of that used by Lowry *et al.* (*4*) for the production of cirrhosis in rats—is not favorable for long survival. After varying periods on the diet, mice became anemic and suddenly died. On the other hand, a few continued to live for long periods with no apparent difficulty except slow growth and small size. In one experiment two mice were sacrificed after 437 days, and in another two were sacrificed after 444 days. The livers of all the mice that survived the first few weeks showed considerable fibrosis, loss of normal architecture, and con-

tained ceroid. Of nine mice that survived more than 200 days, three had hepatomas. The growth of the mice fed this diet is shown in text-figure 2.



TEXT-FIGURE 2.—Weights of three strain BUA mice placed on low-protein, choline-deficient diet at 19 days of age. ●—♀ 31991 had a hepatoma when sacrificed at 444 days of age.

When 50% Ruffex replaced the 50% bentonite of the 50/50 bentonite-basal diet, the mice showed satisfactory growth and no hepatomas developed (table 1). In one experiment the daily food intake per mouse on the following diets was determined: basal, 3.37 gm.; 50/50 bentonite-basal, 6.14 gm.; and 50/50 Ruffex-basal, 5.92 gm. Since half the dietary intake on the last two diets was not food, the actual food intake was as follows: basal, 3.37 gm.; 50% bentonite, 3.07 gm.; and 50% Ruffex, 2.96 gm. Thus, slight inanition may have resulted from both the diets containing 50% bulk-former. This may account for the growth rate of the animals fed the 50/50 Ruffex-basal diet being slightly below the controls, but would not explain the extreme retardation of growth of the animals fed the 50/50 bentonite-basal diet.

Discussion

The original intention of the experiments in which a large amount of bulk-former is included in the diet was to produce sustained inanition with the hope of extending previous studies (*6*). The decreased food intake and decreased growth rate of the mice fed the 50/50 Ruffex-basal diet were only slight, and were accompanied by no discernible cytologic

changes in the liver. The extreme retardation on the 50/50 bentonite-basal diet, on the other hand, was accompanied by the development of fatty livers and eventually of considerable fibrosis and ceroid, and ultimately of hepatomas. The decrease in food intake, at least during the initial period of the experiment, was not more pronounced than on the 50/50 Ruffex-basal diet. The cytologic changes must then be due to some factor other than inanition, and we have shown that apparently choline and probably other dietary deficiencies may be involved (*1*). Furthermore, from the work of Tannenbaum and Silverstone (*7*), it is clear that moderate inanition, rather than producing hepatomas in mice, delays the development and decreases the number of spontaneous hepatomas and azo-dye-induced hepatomas.

The production of hepatomas in rats by feeding diets low in choline and available methyl groups has been extensively studied by Engel and his coworkers (*2*). We have reported the occurrence of hepatomas in mice on diets low in protein and deficient in choline (*3*). This has been further corroborated, with the same diet, in the present study. The addition of as little as 10 percent bentonite to the low-protein, choline-deficient diet prevents growth entirely (*1*). The addition of choline to the 50/50 bentonite-basal diet (*1*) prevents the fatty livers usually produced on this diet and permits better although not normal growth. The evidence, then, indicates that choline is removed from the contents of the intestine by bentonite and that a large amount of bentonite in the diet produces a deficiency in which choline is involved. This may account, in part at least, for the development of hepatomas on the 50/50 bentonite-basal diet.

Since the addition of choline does not restore growth to a normal level, it would appear that the deficiency is not a simple choline deficiency. Other cationic materials may also be removed by bentonite, thus contributing to the deficiency. This may be of importance in the development of the hepatomas and may account for the fact that they appear with greater frequency with the 50/50 bentonite-basal diet than with the diet low in protein and deficient in choline. The nature of these materials and their role in tumor production are being further investigated.

The occurrence of one hepatoma in a control animal is worthy of special comment inasmuch as this is unique in our experience with mice of this strain (BUC), although a large number of them have been used in long-term experiments. Silverstone *et al.* (*8*) have recently reported an increased frequency of spontaneous hepatomas in C3H mice on synthetic diets compared with those on natural foods. Our control was on a semi-synthetic diet, the "basal" diet of the bentonite experiments, but many other animals on this and on other similar diets have not developed hepatomas, which makes this explanation extremely doubtful. The presence of thorotrast is another factor to be considered. The mouse had lived for over 12 months with the Kupffer cells laden with thorotrast. Thorotrast is radioactive and its use in roentgenography has accordingly been questioned. The only report of carcinogenic action in the liver,

however, is a human case reported by MacMahon *et al.* (*9*) which was not a hepatic-cell tumor but was described as a primary endothelial-cell sarcoma. Furthermore, many mice in our experiments have survived as long or longer than this one without developing hepatomas. Finally, the hepatoma in this case occurred in a liver that showed no other signs of damage (fig. 6), as was also true of the spontaneous hepatomas in C3H mice, whereas the hepatomas in the animals fed the 50/50 bentonite-basal diet were in livers otherwise severely damaged (fig. 5), with considerable fibrosis, ceroid, and loss of normal architecture. We conclude, therefore, that this is a spontaneous hepatoma only fortuitously associated with these experiments.

Summary

Hepatomas developed in 11 of 12 mice fed a semisynthetic diet mixed with an equal volume of bentonite. One tumor developed in 1 of 9 control mice, none in 9 mice ingesting a 50/50 mixture of the basal diet and Ruffex. No tumors developed in 18 mice injected with thorotrast, none in 24 control mice raised on the stock diet, but 3 of 9 mice ingesting a low-protein, choline-deficient diet developed hepatomas.

The livers of mice on the 50/50 bentonite-basal diet were severely damaged, as indicated by considerable fibrosis, ceroid, and loss of normal architecture. The liver damage noted in the group ingesting bentonite is consistent with that expected during prolonged choline deficiency. The removal of choline from the intestinal tract by the bentonite, a base-exchange silicate, is tentatively advanced as a partial explanation for the development of the hepatomas in the mice in these experiments.

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PLATE 2

FIGURE 1.—X-ray picture of strain BUB mouse #30920, after three 0.1-cc. injections of thorotrast in the tail vein and after 167 days on the 50/50 bentonite-basal diet, showing a small tumor in the right lobe of the liver. A similar picture a month before showed no tumor. The tumor shows as an area free from thorotrast. The intestines are seen to be distended by the bentonite diet. Actual size.

FIGURE 2.—X-ray picture of the same mouse after 200 days on the bentonite-basal diet, showing growth of the tumor. Actual size.

FIGURE 3.—Photograph (slightly enlarged) of the same mouse at sacrifice on the 203rd day, showing the tumor.

FIGURE 4.—X-ray picture of strain BUC mouse #23653, after three 0.1-cc. injections of thorotrast in the tail vein and 243 days on the basal diet. The sharp edges of the liver lobes and the homogeneous distribution of the thorotrast are characteristic of the normal liver. Actual size.

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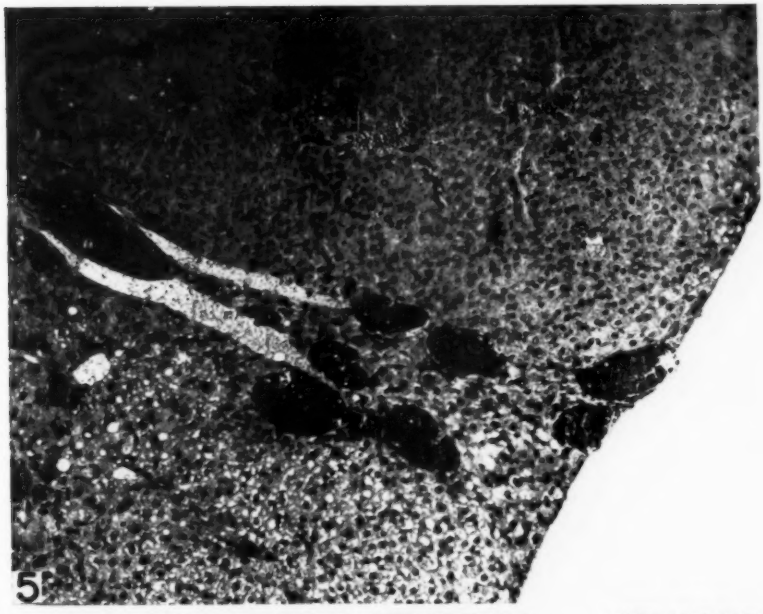
PLATE 3

FIGURE 5.—Photomicrograph of section of the liver of strain BUC male #23659, injected with thorotrast and then placed on the 50/50 bentonite-basal diet for 291 days, showing edge of hepatoma (*above*) and adjacent liver tissue. The round vacuoles in the latter represent fat droplets. The thorotrast is in massive aggregates of macrophages pressed aside by the growing tumor. Delafield's hematoxylin and eosin. 7.5 × ocular and 10 × Zeiss objective.

FIGURE 6.—Photomicrograph of section of the liver of strain BUC female #23657, injected with thorotrast and placed on the basal diet for 375 days. This is the control animal that developed a hepatoma. The liver tissue (*right*) adjacent to the tumor is fairly normal in appearance and in structure. Delafield's hematoxylin and eosin. 7.5 × ocular and 10 × Zeiss objective.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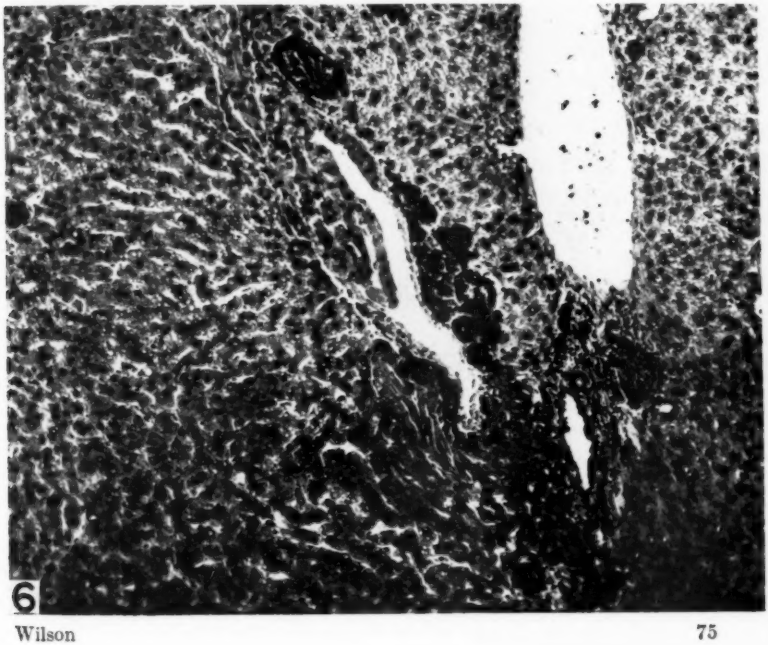
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Induction of Ovarian Neoplasms Following Intrasplenic Transplantation of Ovarian Grafts to Old Castrated Mice[1,2,3]

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The induction of ovarian tumors by intrasplenic transplantation of ovaries into castrated rats or mice is well known. It has been considered that these tumors are the result of a hormonal imbalance with a fall in systemic estrogen and a concomitant rise in the production of gonadotrophin (*1–3*). Li and Gardner (*4*) suggested that a similar endocrine disturbance might be found in aged mice where ovarian secretion has diminished considerably. Since ovarian tumors in intact old mice are encountered infrequently, it was proposed that either the old ovary had lost its ability to respond even to high levels of gonadotrophin or that the secreted gonadotrophin was "inadequate in quality or quantity" (*4*). It was subsequently observed (*4, 5*) that ovaries from old mice are susceptible to tumorigenesis when transplanted intrasplenically into young castrated mice. These results support the possibility that changes in gonadotrophic hormone production or secretion may be responsible for the lack of ovarian tumors in intact old mice. If this were true, one might expect to find no ovarian tumors in old, castrated mice following intrasplenic transplantation of ovarian grafts.

Materials and Methods

Mice from strain BALB/cAn and BALB/cAn×DBA/An F_1 hybrids[4] were employed in these experiments. The animals were housed in an air-conditioned room at a temperature range of 76° to 78° F., and had access to an unlimited supply of Purina laboratory chow pellets and tap water.

Ovaries were excised from strain BALB/c females 50 to 60 days old, and were cleared of fat and each was cut in half. The mice used as hosts were castrated at the start of the experiment. Each castrate was then

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[3] The technical assistance of Lois C. Sumner, Hilda M. Banks, and Dorothy J. Bowland is gratefully acknowledged.

[4] Dr. Howard B. Andervont, Laboratory of Biology, National Cancer Institute, Bethesda, Md., kindly supplied the mice.

implanted intrasplenically with half an ovary, using a 15-gauge short-beveled trocar. Mice bearing palpable tumors or in poor physical condition were sacrificed. Many of the hosts survived 300 to 355 days following transplantation. All mice were examined at autopsy for remnants of gonadal tissue and for splenic adhesions. The accessory sex organs were observed. Both tumor and spleen were excised, fixed in Tellyesniczky's fluid, sectioned at 6 microns, and stained with hematoxylin and eosin. Tumors were diagnosed following microscopic examination.

Experimental Procedures and Results

The halved ovaries from each strain BALB/c donor were transplanted to 4 castrated mice including the donor, a strain BALB/c male, and 2 BALB/c×DBA F_1 hybrid males or females. The strain BALB/c hosts varied in age from 40 to 63 days, while the hybrids varied from 498 to 575 days at the time of transplantation. Old BALB/c×DBA F_1 hybrids were selected in preference to old strain BALB/c mice because it was believed a much higher proportion of the hybrids would survive long enough to permit the development of intrasplenic ovarian tumors. Forty strain BALB/c and 36 BALB/c×DBA F_1 hybrid mice survived 200 or more days. One male and 3 female hybrids were observed with adhesions to the body wall and thus were not included in table 1. As a result, the effective total for the BALB/c×DBA F_1 hybrid group was 32 mice.

The ovarian tumors observed in this experiment included granulosa-cell tumors, mixed tumors containing granulosa and lutein cells in varying proportions, and granulosa-cell tumors that had become luteinized. The results may be found in table 1.

Discussion and Conclusions

A tumor incidence of 81 percent was observed when ovaries from young strain BALB/c mice were transplanted into old BALB/c×DBA F_1 hybrid castrates. When young strain BALB/c ovaries were transplanted into young castrates of the same strain, 85 percent bore tumors (table 1). Thus ovarian tumors were induced as readily in old as in young hosts. It has been suggested that ovarian neoplasms are induced in castrated rats and mice as the result of an increased level of circulating gonadotrophin (*1–3*). If this theory is correct, the appearance of ovarian tumors among old castrated mice in the present experiment would indicate a similar high level of gonadotrophic hormone.

Spontaneous ovarian tumors are infrequent in old intact mice, although the ovaries from these mice are susceptible when transplanted intrasplenically into young castrates. On the basis of these observations, Li and Gardner (*4*) suggested that age changes occurred in the old pituitary gland coincident with those of the old ovary, so that the production of gonadotrophin was no longer adequate in quality or quantity to produce ovarian tumors. This, apparently, was not the condition in the old castrates of the present experiment, however, inasmuch as such tumors

TABLE 1.—*Summary of data on intrasplenic ovarian transplantation in castrated strain BALB/c and BALB/c×DBA F_1 hybrid mice. All transplants were 50 to 60 days old at the start of the experiment.*

Series	No. mice	Strain	Sex	Average age of mice		Average observation period, all mice	Mice with ovarian tumors	Percent mice with tumors	Average tumor size
				at start (*days*)	at autopsy (*days*)	(*days*)			(*mm.*)
A1	16	BALB/c	M	51	341	290	15	94	8×7
A2	24	BALB/c	F	54	368	314	19	79	7×5
Series A (A1+A2)	40	BALB/c	M+F	53	357	304	34	85	7×6
B1	13	BALB/c×DBA	M	558	818	260	13	100	8×6
B2	19	BALB/c×DBA	F	543	834	291	13	68	7×6
Series B (B1+B2)	32	BALB/c×DBA	M+F	549	828	279	26	81	7×6

were induced frequently. Thus, if a change which inhibited ovarian tumorigenesis occurred in the pituitary gland of old intact mice, one would have to conclude this was modified or reversed by castration. It may be that the anterior lobe of the pituitary gland of old intact mice becomes hyperactive as ovarian function declines, and that it does not remain so. It also is possible that the concentration of circulating gonadotrophin never reaches a sufficiently high level to be effective in ovarian tumorigenesis because the old gonad may still, to some degree, be able to restrain activity of the anterior lobe of the pituitary gland. Smith and Gardner (*6*) transplanted pituitary glands from mice of varying age and concluded that the gonadotrophin content as reflected in uterine weight response was higher in old castrated mice than in intact mice of the same age—also, that this content reached a peak in intact mice at 500 to 600 days and then diminished. If a continued high level of circulating gonadotrophin is required in ovarian tumorigenesis, this could explain the lack of spontaneous ovarian tumors in old intact mice. In support of this assumption, it has been observed that repeated injections of estrogen or androgen into young castrated mice bearing intrasplenic ovarian grafts will inhibit ovarian tumorigenesis completely even though hormone therapy is begun 96 to 138 days following transplantation (*7*). In the present experiment, 93 percent of the old hybrids were castrated when 500 to 600 days of age. The fact that ovarian tumors subsequently were induced in many of these mice indicates that a continuous high level of circulating gonadotrophin probably was maintained.

Despite a smaller average period of observation, the incidence of ovarian tumors in castrated male mice appeared significantly higher than that of castrated female mice in both series A and B (table 1). What this difference may be attributed to is not known, although it may be related to a sex difference in gonadotrophin production and secretion.

At autopsy, an average tumor size of 7 mm.$\times$ 6 mm. was observed in both young and old castrates (series A,B—table 1). All the mice had been implanted initially with young ovaries of a similar age and, presumably, about the same proliferative capacity. The average period of observation for series A and B was 304 and 279 days respectively. Assuming no appreciable change in tumor size in series B due to the few days' difference in observation period, the results suggest that the growth of ovarian tumors within the spleen of castrated mice is little influenced by the age of the host.

Summary

Strain BALB/c mice and BALB/c$\times$DBA F_1 hybrid mice averaging 53 and 549 days of age respectively, and of both sexes, were gonadectomized and on the same day implanted intrasplenically with ovarian grafts derived from 50- to 60-day-old strain BALB/c mice. The castrated hosts were autopsied after an average observation period of 304 days for strain BALB/c and 279 days for the BALB/c$\times$DBA F_1 hybrids. Among those that survived over 200 days and had no splenic adhesions, 34 of 40 young

mice from strain BALB/c and 26 of 32 old castrated mice from the hybrid group bore ovarian tumors. The lack of spontaneous ovarian tumors in old, intact mice is discussed in the light of this observation.

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Influence of Continued and Intermittent Painting with Croton Oil on Skin Tumorigenesis in Mice [1,2]

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It has been observed repeatedly in mice that a single painting with a carcinogen is inadequate, in most instances, to induce skin tumors. These tumors, however, are observed if the noncarcinogen, croton oil, is subsequently applied to the same site (*1-3*). Since skin tumors appeared even when treatment with croton oil was begun 43 weeks after application of the carcinogen, it was concluded that the skin changes produced by the carcinogen probably were irreversible (*4*). In contrast to this, it has been observed that considerably more than one application of croton oil is required for effective promoting action, and that the cocarcinogenic influence of this compound is reversible (*5,6*). The mice employed in the latter experiments were painted repeatedly with croton oil following which treatment was suspended for 9 to 20 weeks and then resumed. In view of the long period of nontreatment, it was considered of interest to determine whether or not the promoting effect could be similarly reversed using as short an interval as 2 weeks between applications of croton oil.

Although the ability of croton oil to promote the development of visible skin tumors in mice is well known, the influence of this compound on tumors already induced has not yet been fully established.

In addition to the skin tumors that first appear in response to painting with croton oil, there undoubtedly are others in the tumor-bearing mice that have not yet attained a visible size. Would the latter growths develop into visible skin tumors in the absence of further treatment with croton oil, or would this require the continued presence of promoter? These and related problems have been investigated and are reported in the present communication.

Materials and Methods

Strain DBA/2Jax male mice, 50 to 70 days of age, were painted on both ears with a 0.5 percent solution of 20-methylcholanthrene [3] and a 5.0 per-

[1] Received for publication February 9, 1953.
[2] This investigation was supported by a research grant from the Damon Runyon Memorial Fund.
[3] M. p., 179°-180° C., referred to hereafter as MCA.

cent solution of croton oil[4] in olive oil. Both solutions were applied in similar fashion with one stroke of a No. 3 camel's-hair brush. After application of the carcinogen, the mice were isolated one to a cage for several days to minimize loss of the compound through licking and rubbing, and then were regrouped and housed ten to a cage. The mice were examined once weekly, starting several weeks after the first painting with croton oil. Although all observable growths were recorded, none was considered a tumor unless it persisted at least 2 weeks and attained a minimum size of 1 mm. in diameter. At autopsy, the ears of each mouse were excised and fixed in Tellyesniczky's fluid. Whenever possible, tumor diagnoses were confirmed by microscopic examination.

Experimental Procedures and Results

The experiment was divided into two sections:

Section I.—72 mice received one application of methylcholanthrene (MCA) followed, after an interim of 2 weeks, by paintings twice weekly with croton oil. Those mice in which skin tumors appeared were divided into 2 groups: Group A, in which treatment with croton oil was continued, and Group B, in which it was discontinued. To minimize variability, the tumor-bearing mice were distributed alternately to Groups A and B. Thus, the first mouse bearing a tumor was placed in Group A, the second in Group B, the third in Group A, the fourth in Group B, etc. All surviving mice were sacrificed 6 weeks following the last (50th) application of croton oil.

Section II.—30 mice were painted with MCA as described previously. Painting with croton oil was begun 20 days later and was continued once every 2 weeks for the remainder of the experiment except for a period of 8 weeks following the 7th application when, through an oversight, treatment was suspended.

The tumor-bearing mice had one skin tumor each when apportioned to Groups A and B. The influence of continued or discontinued painting with croton oil on these tumors is summarized in table 1. It will be observed that 13 (68 percent) of the tumors regressed in Group A, and 8 (53 percent) in Group B. Statistical analysis (by the chi-square test) indicated no association between continued or discontinued treatment and the tendency for tumors to regress or be sustained. The average regression time was 55 days in Group A and 40 days in Group B. Statistical analysis (Students' t test) showed no significance when these times were compared. Maximum tumor size, a measure of the largest diameter observed for each tumor, was recorded and the means computed for Groups A and B. The difference between the means was slight (table 1).

The mice in Group A received an additional 16 paintings with croton oil, after the first tumors appeared, for an average total of 49 applications. Ten new tumors were observed in 5 of the mice in Group A compared to 4 in 4 of the mice in Group B (table 2). Five of these tumors in Group

[4] Obtained from the Fisher Scientific Co., Eimer and Amend, New York, N. Y.

TABLE 1.—*Influence of continued versus discontinued application of 5 percent croton oil on skin tumors induced with methylcholanthrene and croton oil. The mice had one tumor each when apportioned to Groups A and B.*

Group	No. mice with tumors	No. applications croton oil, average		Total no. tumors	Sustained tumors observed, average (*days*)	No. tumor regressions	Period before tumors regressed, average (*days*)	Latent period, average* (*days*)	Tumor size, average† (*mm.*)	Period of observation, average‡ (*days*)
		before tumors appeared	after tumors appeared							
A	19	33	16	6	93	13	55	110	3.1	221
B	15	31	0	7	98	8	40	104	2.8	220

*Measured from first painting with croton oil.
†A measure of the largest diameter observed for each tumor.
‡Measured from painting with MCA.

A were sustained, and 2 in Group B. The time taken for the remaining tumors to regress did not differ appreciably from that previously reported for regresssions in table 1. The average latent period for the new tumors, measured from the time the mice were segregated into Groups A and B, was 44 and 63 days respectively. Statistical analysis (by the chi-square test) showed no association between continued treatment with croton oil and the length of the latent period. The early and the later appearing tumors were found with about the same degree of frequency in both series. The means for tumor size were comparable to those reported for the tumors that arose earlier (table 1). No new tumors were observed in either group following the last painting with croton oil.

TABLE 2.—*Late-developing tumors resulting from continued versus discontinued application of 5 percent croton oil on skin tumors. The mice had one tumor each when apportioned to Groups A and B.*

Group	No. mice with tumors	Mice developing additional tumors*	No. additional tumors*	No. additional applications croton oil, average	Latent period, average† (*days*)	Tumor size, average‡ (*mm.*)	Period of observation, average† (*days*)
A.....	19	5	10	16	44	2.3	94
B.....	15	4	4	0	63	2.1	103

* Observed subsequent to division of mice into Groups A and B.
† Measured from time of division of mice into Groups A and B.
‡ A measure of the largest diameter observed for each tumor.

There were 22 survivors at the time the first tumor was observed in Section II, in which the mice were painted intermittently with croton oil (table 3). A total of 8 tumors was observed in 6 mice after a mean latent period of 137 days measured from the start of the second series of paintings with croton oil. One of the tumors regressed in 147 days, while 7 were sustained. The latter had been observed for an average of 130 days when the experiment was terminated.

The tumors observed in the present experiment were benign papillomas that varied in size from 1 mm. to 6 mm. in their largest diameter. Generally the smaller ones were sessile, while the larger ones were broad-based and fleshy. The benign nature of these tumors was confirmed histologically whenever possible. A representative example of the broad-based type is shown in figure 1.

Discussion and Conclusions

The percent of tumors that regressed or were sustained under continued treatment with croton oil did not differ significantly when treatment was discontinued. Comparison of the mean time for tumors to regress or the mean for tumor size in Groups A and B also was not significant (table 1). These results indicate that continued painting of induced skin tumors with croton oil has no apparent influence on subsequent growth or regres-

TABLE 3.—*Influence of intermittent application of 5 percent croton oil on skin tumorigenesis following a single application of 0.5 percent methylcholanthrene. The croton oil was administered once every 2 weeks throughout the experiment except for a period of 8 weeks beginning with the 7th application.*

Survivors at time of first tumor	Total applications croton oil, average	No. mice with tumors	Tumor incidence (*percent*)	Tumor size, average* (*mm.*)	Latent period, average		No. tumors sustained	No. tumor regressions	Period of observation, average§ (*days*)
					From croton oil no. 1† (*days*)	From croton oil no. 2‡ (*days*)			
22	24	6	27	4.0	281	137	7	1	402

*A measure of the largest diameter observed for each tumor.
†Measured from start of painting with croton oil.
‡Measured from start of second period of painting with croton oil.
§Measured from application of MCA.

sion. Although Berenblum (*7*) reported that continued application of croton resin, the active principle of croton oil, facilitated the transformation of benign tumors to malignant tumors, this was not the case in the present experiment in which no malignant tumors were observed. These two experiments are not strictly comparable, however, since Berenblum (*7*) applied carcinogen weekly up to the time of appearance of a tumor whereas the mice we employed received only one application of methylcholanthrene. The fact that most tumors induced with one low dose of a carcinogen and repeated applications of croton oil are benign (*3*, *8–10*), suggests the need for applying a greater dose of carcinogen initially, before a promoting effect of croton oil on the malignant transformation may be demonstrated. Thus, it already has been reported that more malignant skin tumors are induced in mice following painting with one massive dose of MCA (*9*).

While skin tumors were obtained in the mice in Groups A and B in response to painting with croton oil (table 1), other foci capable of developing into visible tumors doubtless existed in the treated tissue. Thus additional tumors arose when the period of painting with croton oil was extended (Group A, table 2). The appearance of skin tumors in Group B (table 2), however, indicates for these tumors, at least, an ability for continued development in the absence of further promotion with croton oil. In a related experiment in cocarcinogenesis, Salaman (*5*) proposed a "critical size, rather less than 1 mm. diameter" below which the addition of more croton oil was required for the production of visible tumors. Although the tumors in Group B (table 2) may have conformed to this suggested size, no data are available to confirm this possibility. The lack of a significant difference in tumor incidence, latent period, or subsequent tumor development between the mice in Group A and those in Group B (table 2) was unexpected. It may be that a maximum or near-maximum promoting effect already had been attained when the tumor-bearing mice were apportioned to Groups A and B. Thus, in comparable experiments in which a 5 percent solution of croton oil was also employed, an average of about 2 tumors per mouse was observed even though the mice received as many as 70 to 80 applications (*3*, *8*). It has been reported recently that many more skin tumors per mouse are obtained when a 2.5 percent solution of croton oil is employed (*5*). On the basis of these results, it can be concluded that for accurate determination of the role of continued painting on the production of skin tumors in mice in which such tumors already have been induced, one should employ a solution of croton oil at a concentration of 2.5 percent or possibly less.

In experiments on the influence of interrupted treatment with croton oil on skin tumorigenesis, it had been observed that the promoting influence of this compound could be reversed if treatment was suspended 9 weeks or longer (*5*, *6*). In Section II in the present experiment, croton oil was applied once every 2 weeks. If the promoting effect of croton oil were similarly reversed under these conditions, one would not expect to obtain skin tumors. This was not observed, however, for 6 of the 22 survivors were susceptible (table 3). Thus, by shortening the time interval between successive applications of croton oil to 2 weeks, a cumulative promoting effect with the development of visible tumors was demonstrated. However, the average latent period was longer when croton oil was applied at intervals of 2 weeks than when applied twice weekly.

Summary

Skin tumors were induced in strain DBA mice that had been painted once on the ears with methylcholanthrene followed by repeated painting with croton oil. The tumor-bearing animals were divided into 2 groups: in one, treatment with croton oil was continued, while in the other it was discontinued. It was observed that continued treatment did not influence subsequent growth or regression of the tumors already induced.

Some of the tumor-bearing mice bore additional tumors during the course of continued treatment with croton oil. The fact that new tumors were also observed among the tumor-bearing mice no longer exposed to croton oil indicates, for these tumors at least, that once a certain stage in tumor promotion has been reached, further application of croton oil is unnecessary to obtain visible tumors.

In another group, strain DBA mice were painted with methylcholanthrene as before but croton oil was applied once every 2 weeks. Skin tumors were induced in 27 percent of the survivors, indicating a cumulative promoting influence despite repeated periods of nontreatment.

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PLATE 4

FIGURE 1.—Papilloma of the skin induced in mouse with one application of methylcholanthrene and continued application of croton oil. Hematoxylin and eosin. × 100

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A Comparison of the Recovery of Isotopic and of Diazotizable Nitrogen Following Gastric Intubation of 2-Acetylaminofluorene-N^{15} to Rats [1,2]

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Studies in this laboratory, using a quantitative diazotization method of analysis of the tissues and excreta of rats, yielded incomplete recoveries often amounting to as little as 30 percent of that part of the orally administered 2-acetylaminofluorene (AAF) that had disappeared from the stomach (*1*, *2*). Even after hydrolysis of proteins and of lipids of the tissues no significant increase was obtained in the amount of diazotizable nitrogen recovered (*2*). It was decided, therefore, to follow the fate of the carcinogen by determining the N^{15} distribution after administration of AAF enriched with N^{15}.

If more nearly quantitative recovery of administered AAF could be obtained by analyzing the tissues and excreta for isotopic rather than for diazotizable nitrogen, it might be interpreted: 1) that a large portion of the nitrogen of AAF is removed from the molecule during metabolism; or 2) that the nitrogen of aminofluorene (AF) or AAF becomes conjugated in some fashion that prevents its diazotization; or 3) that the molecule is changed in such a way that the chromogen in the diazotization method (*3*) no longer has the same optical density as that formed from AF.

Gutmann, Kiely, and Klein (*4*) have recently suggested that the apparent disappearance of aromatic amino groups of AF and AAF in the rat may be due to *in vivo* hydroxylation of the compounds. Gutmann (*5*) found that sodium 7-hydroxyfluorenyl-2-azo-2′-naphthol-3′,6′-disulfonate, the chromogen from 7-hydroxy-2-aminofluorene (7-OH-AF) in the diazotization method, absorbs maximally at the same wave length as that of sodium fluorenyl-2-azo-2′-naphthol-3′,6′-disulfonate, the chromogen from AF, but the extinction due to the former was found to be only 37 percent of that given by the latter. Some of the observations made in this laboratory support the supposition that the low recoveries obtained with the diazotization method are due *in part* to the presence of the hydroxy derivative as a metabolite of the administered AAF. The

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metabolite 7-OH-AAF, however, does not account for all of the difference between the recoveries by the diazotization and by the N^{15} methods of analysis.

Since recovery studies of AAF based on radioactivity measurements, using C^{14} in position 9 as a tracer, have been carried out by Morris, Weisburger, and Weisburger (*6, 7*), a comparison of the results with N^{15} in position 2 and C^{14} in position 9 affords strong suggestive evidence for the intactness of the molecule of AAF during metabolism with respect to these two positions.

Materials and Methods

Young adult Buffalo rats were used (*8*). During the experimental period they were kept in individual metabolism cages and were fed a diet of natural foodstuffs, Morris No. 222, *ad libitum*. This diet contains approximately 22 percent protein from several sources, and 10 percent fat. Water was always available. 2-Acetylaminofluorene,[4] containing 60 atom percent excess N^{15}, was administered by stomach tube in a single dose of 10 to 20 mg.—or repeatedly in doses from 1.3 to 10 mg.—per 100 gm. of body weight. The material was dissolved in propylene glycol except for rats 8, 9, and 10 for which coconut oil was used.

For the studies on bile, fistulas were made by introducing polyethylene tubing into the common bile ducts of rats under ether anesthesia.[5] Small samples of control bile were collected, after which AAF was administered by gastric intubation. During the period of collection of bile the rats were force-fed daily an emulsion of casein, milk powder, and dried ox bile. Saline and glucose solutions were administered subcutaneously twice daily.

At intervals of 2, 6, and 18 hours, or 2, 3, and 4 weeks after the last of repeated doses of AAF, the animals were etherized and bled to death by heart puncture. The blood, urine, feces, and bile, and various lyophilized tissues of the rat were analyzed for total nitrogen and for N^{15}. The micro-Kjeldahl procedure, using for digestion H_2SO_4, $CuSO_4$, K_2SO_4, and one Hengar selenized granule, was found to yield complete recovery of the nitrogen of AAF and was therefore employed for total nitrogen determinations. Following alkalization, the ammonia was distilled from the digestion tube into 4 percent boric acid solution and was titrated with 0.01 N HCl using a mixed indicator (ratio of 1 : 5) of 0.1 percent alcoholic methyl red and 0.1 percent alcoholic bromocresol green. Each sample was concentrated to approximately 5 ml., the ammonium nitrogen was oxidized to nitrogen by sodium hypobromite, and the nitrogen gas was collected in tubes that could be sealed off and stored.

The N^{15} : N ratios were determined in the Consolidated-Nier mass spectrometer.[6] Correction was made for the N^{15} : N ratios found for corresponding materials from untreated control rats. The recoveries based on N^{15} analyses are conservative since the atom percent excess of N^{15} found in control material varied from .0035 to .006, and the highest value (0.006) found in the controls was routinely subtracted from the atom percent excess N^{15} of the material from treated animals in estimating the total N^{15} content. This correction, which has little effect on the results in samples having high N^{15} : N ratios, makes a significant difference when the ratios are relatively small and the amounts of total nitrogen are large.

Acetone extracts of tissues of some of the experimental animals, after removal of the acetone at room temperature, were analyzed for AAF by N^{15} recovery and by the

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[4] 2-Acetylaminofluorene-N^{15} was prepared for these experiments by Argus and Ray (*9*) in the Cancer Research Laboratory, University of Florida, Gainesville, Florida, on a U. S. Public Health Service grant No. C.

[5] The bile-fistula animals were prepared by Mrs. Helen Ross Stecklein and Mrs. Billie P. Wagner.

[6] The authors are indebted to Mr. William Comstock of the National Cancer Institute for the analyses in the mass spectrometer.

method for "specific" [7] diazotizable material. In some cases the acetone extracts, after evaporation of the solvent, were taken up in ether and water, transferred to separatory funnels, and the layers were separated after three extractions of the water layers with ether. Some samples of urine were extracted with ether. The pooled aqueous and pooled ether layers were analyzed separately for isotopic and for diazotizable nitrogen.

For the data reported in this paper the material found by the "specific" diazotization method was calculated as AAF using a standard curve of AAF. If the hydroxy derivatives were present in the material analyzed, the calculated values would thus be lower than the amount of AF or AAF and 7-OH-AF and/or 7-OH-AAF actually present, since 7-OH-AF produces considerably less color than AF in the "specific" diazotization test (*5*). Since a method has recently been developed in this laboratory by Damron (*10*) for the colorimetric determination of 7-OH-AF in the presence of AF and AAF, it has been used in a few instances to correct the data obtained by the diazotization method. The corrected results have been considered [8] in discussing the difference in recoveries of AAF and its metabolites by the diazotization method and by analysis of N^{15}.

Experimental Results and Discussion

Total recoveries.—The data in table 1 demonstrate the greater recovery of N^{15}-calculated AAF from the tissues and excreta of rats than of "specific" diazotizable material after gastric intubation of 2-AAF-N^{15}. Exclusive of the AAF still present in the gastrointestinal tract, the total recoveries calculated from isotopic nitrogen are two to three times greater than those obtained by the diazotization method (table 1, last column). It should be noted (table 1), however, that recoveries from the gastrointestinal tract, when there is considerable unabsorbed AAF present, are not significantly different by the two methods of analysis.

Recoveries from the tissues.—Qualitatively there is similarity in the distribution of N^{15} and of diazotizable material in the tissues. As previously found with the diazotization method (*1*, *2*), and more recently reported for 2-AAF-9-C^{14} (*6*, *7*), the amount of material recoverable from the tissues decreases while the major portion of the AAF may still remain in the stomach. Text-figure 1 and table 1 illustrate this phenomenon. Continuous administration of AAF-N^{15} does not result in the accumulation of N^{15} in the tissues, at least in concentrations that are detectable. Rats 8, 9, and 10 were killed 2, 3, and 4 weeks after the 48th gastric intubation of 2-AAF-N^{15} made at the rate of three intubations per week. No N^{15} was found in the lyophilized tissues of these animals.

Recoveries from excreta.—Eighty-three, 83, and 81 percent of the total amounts of N^{15} administered (98, 99, and 103 mg. of AAF-N^{15} respectively) were recovered from the urine and feces of rats 8, 9, and 10. Following the last dose of AAF, N^{15} was present in the urine in decreasing amounts

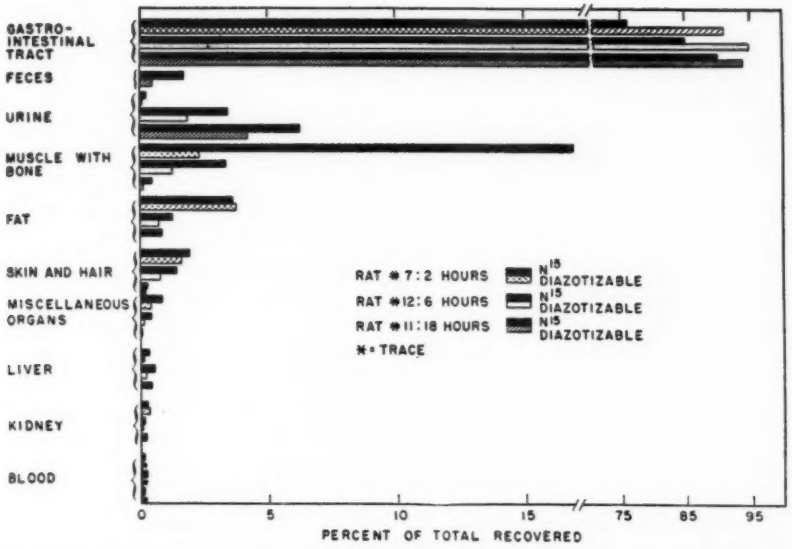
[7] "Specific" diazotizable material is the material that reacts with R salt to give a chromogen having the same absorption spectrum as that of 2-fluorenyl-azo-1-naphthol-3,6'-disulfonic acid sodium salt (*5*).

[8] It has been assumed that the material estimated by Damron's method is 7-OH-AAF or 7-OH-AF, although it is recognized that there may be present some other metabolite of AAF which will be found to give the reaction.

TABLE 1.—*The recovery of N^{15} and of "specific" diazotizable material following gastric intubation of 2-acetylaminofluorene-N^{15}*

Rat No.	Dosage		Time of survival	Percent of administered acetylaminofluorene*												Percent of N^{15}-calculated AAF (exclusive of G. I. tract contents) accounted for by diazotization method
				Gastrointestinal tract		Urine		Feces		Bile		Tissues		Total		
	Mg. per dose	*No. of doses*	*Hours*	N^{15}	*Diazo*	N^{15}	*Diazo*	N^{15}	*Diazo*	N^{15}	*Diazo*	N^{15}	*Diazo*	N^{15}	*Diazo*	
7.....	42	1	2	75	82	0.3	0.16	—	—	—	—	24	7	99	89	29
1.....	17	1	2	60	—	0.5	—	1.0	—	—	—	25	—	87	—	—
12.....	45	1	6	72	75	2.5	1.4	—	—	—	—	8	2.6	83	79	36
11.....	47	1	18	84	88	6	4	1.4	0.6	—	—	2	0.4	94	93	53
2,B.F.†..	18	1	24–40	44	38	10	4	4	2	22	17	10	2	91	63	54
3,B.F.†..	19	1	27–32	49	55	7	2	0.7	0.2	12	6	10	2	79	65	33
5,B.F.†..	10	8	(324)‡60	2	1	46	20	28	2	3	2	2	1	81	26	32
6,B.F.†..	9	8	(336)‡44	1.5	0.8	48	23	29	2	9	6	1	0.3	88	31	35

*Recoveries calculated as acetylaminofluorene.
†B. F.: Biliary fistulas introduced before single dose, or final of repeated doses, of carcinogen.
‡Figures in parentheses represent period between first of repeated administrations of carcinogen and time of analysis of tissues, while the figures not in parentheses represent time of survival following the introduction of the bile fistula and administration of the final dose of acetylaminofluorene.



TEXT-FIGURE 1.—Comparison of distribution of acetylaminofluorene calculated from N^{15} and from "specific" diazotizable material 2, 6, and 18 hours after administration of 2-acetylaminofluorene-N^{15}.

TABLE 2.—*Distribution of excreted N^{15} between urine and feces of rats during repeated gastric intubation or feeding of 2-acetylaminofluorene-N^{15}*

Rat No.	Percent of total	
	Urine	Feces*
1 C†	65	35
2–11 C (pooled)†	68	32
8, per. I–IV‡	65	35
9, per. I–IV‡	62	38
10, per. I–IV‡	63	37
19 and 20§	64	36
21 and 22§	56	44
23 and 24§	62	38
25§	70	30
26 and 27§	71	29

*The metabolism cages are constructed so that contamination of the feces with urine is usually, but not always, prevented.

†Rats 1 C and 2–11 C received repeated doses of 10 mg. AAF-N^{15} per 100 gm. body weight, 3 times weekly for 4 weeks.

‡Rats 8, 9 and 10: periods I to IV cover the 16 weeks during which 1.3 mg. AAF-N^{15} per 100 gm. body weight was administered 3 times weekly.

§Rats 17 through 27 received Morris diet 222–1, containing 0.25 mg. AAF per gm. food, for 6 months. During the collection period of the last 10 days AAF-N^{15} was substituted for AAF in the diet.

for 48 to 72 hours, and in the feces traces of N^{15} were detectable for 8 to 9 days.

As shown in table 2, during repeated gastric or oral administration of AAF-N^{15} to rats, from 56 to 71 (average 65) percent of the N^{15} recovered from the excreta is found in the urine, and from 29 to 44 (average 335

percent in the feces. The recoveries of N^{15} from urine and feces are similar to those reported for C^{14} following gastric administration of 2-AAF-9-C^{14} (7).

The diazotization method sometimes presents difficulty in the analysis of urine due to interference by urinary pigment, resulting in low recoveries. The amount of interference varies inversely with the concentration of diazotizable material, since the greater the concentration of the latter the more the urine may be diluted. Therefore, the degree of interference by urinary pigment, in the analysis of diazotizable material in the urine, is related to the dosage of AAF administered. Attempts to rid the urine of pigment without adsorption of the diazotizable and the N^{15}-containing material have been unsuccessful. Table 3 presents a comparison of recoveries of N^{15} and of diazotizable nitrogen, expressed as AAF, from the urine of rats after the administration of AAF-N^{15}. Recoveries by the diazotization method varied with different doses and schedules of administration from 31 to 69 (average 46) percent of those by N^{15} analyses. The pooled urine of rats 2–11 C was available for analysis by the method developed for 7-OH-AF. If the recovery of diazotizable material is corrected for the lower color production of the hydroxy derivative in the diazotization method, 65 percent instead of 48 percent of the N^{15} can be accounted for by diazotizable nitrogen.

TABLE 3.—*Comparison of recoveries of N^{15} and of "specific" diazotizable nitrogen from urine of rats following gastric administration of 2-acetylaminofluorene-N^{15}*

Rat No.	Acetylaminofluorene by N^{15} method* (*mg.*)	Percent of N^{15} AAF accounted for by diazotization method
11†	2.7	69
12†	1.1	53
1 C‡	68.6	45
2–11 C (pooled)‡	869	48
6§	30.9	39
8 per. V‖	1.5	32
9 per. V‖	1.4	31
10 per. V‖	1.2	49
9 per. I–IV‖	33.4	50

*Recoveries of N^{15} are calculated as AAF.
†Rats 11 and 12 received single doses of 20 mg. AAF-N^{15} per 100 gm. body weight. They were sacrificed at 18 and 2 hours, respectively, following administration.
‡Rats 1 C and 2–11 C received repeated doses of 10 mg. AAF-N^{15} per 100 gm. body weight, 3 times weekly for 4 weeks.
§Rat 6 received 7 repeated doses of 5 mg. AAF-N^{15} per 100 gm. body weight during 12 days.
‖Period V is the 24-hour period after the last dose of 1.3 mg. AAF-N^{15} per 100 gm. body weight administered 3 times weekly for 16 weeks. Periods I to IV cover the 16 weeks.

The samples of urine listed in table 3 were preserved by the addition of HCl to the collection flasks. In another experiment in which $Na_2S_2O_4$ was used as a preservative for the collection of urine of one group of rats (Nos. 17 to 27), it was found necessary to neutralize the urine before analysis by the diazotization method. After neutralization there was interference with the quantitative recovery of added AAF, and the

amount of diazotizable material found in the urine of these rats accounted for only 20 to 35 (average 26) percent of the recoverable N^{15}. This observation suggests that $Na_2S_2O_4$ should not be used as a preservative if the urine is to be analyzed for diazotizable nitrogen.

The diazotization method has proved to be even less satisfactory for the analysis of feces, since it has been found impossible to extract the N^{15}-containing material quantitatively from the feces by any of the many solvents tested. Since the analyses for isotopic nitrogen may be made on the dry feces, while the diazotization method requires a suitable solution, a comparison of total recoveries of fecal AAF or its metabolites by the N^{15} and diazotization methods is of little value. Acetone, used in the manner prescribed for the extraction of tissues (*1*), removes from 25 to 40 percent of the total N^{15} of the feces, and repeated drying and re-extraction will yield small additional amounts. Use of 92 percent acetone instead of 100 percent increases the extractable N^{15} to approximately 50 percent of the total. Repeated extractions with hot water remove about 25 percent of the N^{15}, and again, repeated drying and re-extraction will yield a total of approximately 40 percent of the original N^{15}. Hot glacial acetic acid, used repeatedly, will extract as much as 75 percent of the N^{15} but it also extracts interfering pigment, 35 percent of the total nitrogen, and 43 percent of the total solids of the feces.

The results of the analysis of the acetone extract of the pooled feces from rats 2–11 C (see table 3, footnote ‡) were as follows. Fifty percent of the N^{15} of the feces was extracted by 92 percent acetone. Approximately one-third of the acetone-extracted N^{15} was accounted for by the diazotization method. When correction was made for the lesser color production of the 7-OH-AAF assumed [8] to be present, the total amount of diazotizable material in the acetone extract accounted for 54 percent of that recovered from the extract as N^{15}. Thus it appears that some of the "hydroxy" [8] derivative is present in the feces and that a large portion of the acetone-extractable N^{15} of the feces is not diazotizable.

Recoveries from bile.—In spite of forced feeding, none of the rats with bile fistulas remained healthy in appearance and all of them died between 17 and 67 hours after administration of AAF-N^{15}. However, the results of these studies, presented in table 4, support the thesis that bile is a major excretory pathway for AAF or its metabolites. The greatest amounts of N^{15} and of diazotizable material appear in the bile during the first 24 hours following oral administration of the carcinogen. This observed peak of excretion parallels that found in the urine of intact rats. During the second day there is continued excretion through the bile but the amount of AAF is much less than that which appears during the first day. This decrease in the amount of N^{15} appearing in the bile after 24 hours is observed even when more than 50 percent of the administered AAF remains in the stomach, and it does not appear to be related to a decrease in the rate of flow of the bile. The data do not permit any conclusions as to the effect of the loss of material through the bile fistula

[8] See footnote 8, page 95.

TABLE 4.—*Comparison of the recoveries of N^{15} and of "specific" diazotizable material from the bile and urine of bile-fistula rats after a single gastric intubation of 2-acetylaminofluorene N^{15}**

Rat No.	Total amount administered (*mg.*)	Period of collection following time of administration of AAF (*hours*)	Volume of bile (*ml.*)	AAF recovered from bile		AAF recovered from urine	
				Calculated from recovered N^{15} (*μgm.*)	Percent of N^{15} AAF accounted for by diazotization method	Calculated from recovered N^{15} (*μgm.*)	Percent of N^{15} AAF accounted for by diazotization method
2	18	>24<40	15	3655	76	1682	43
3	19	>27<32	12	2198	55	1310	25
5	10	0–27	7	1717	70	.....	.....
"	—	27–50	17	292	29	.....	.....
"	—	50–<67	5	123	63	3431	74
6	9	0–24	18	5574	68	.....	.....
"	—	24–44	11	582	39	1860	83
33	40	0–17	>6	1312	55	1554	71
34	25	0–16.5	2.5	659	56	.....	.....
"	—	16.5–<40	—	207	55	1742	56
35	32	0–22	5.4	840	53	978	40
"	—	22–46	6.5	311	0	420	50
36	33	0–17	7.2	1714	63	930	48
"	—	17–25	2.8	564	68	.....	.....
"	—	25–36	3.4	335	51	1419	62

*Recoveries are expressed as AAF calculated from N^{15} and by the diazotization method respectively.

on the amount excreted by the kidneys, but it is apparent that considerable N^{15} and diazotizable material are excreted by the kidneys during the time they are being excreted through the bile fistula.

A comparison was made of the amounts in the bile and urine and in the fecal material from the rectum of four rats (Nos. 33 to 36) of AAF calculated from N^{15} ratios, with that determined by the diazotization method. Single doses of 20 or 25 mg. of AAF-N^{15} per 100 gm. of body weight were administered to the rats but absorption of the AAF appeared to be unusually slow. The feces contained so little of the administered N^{15}, compared with the bile and urine, that the analyses are not included in the table. It is evident from the data presented in table 4 that the recoveries of diazotizable nitrogen account for considerably less of the carcinogen than do those of isotopic nitrogen in both bile and urine. Preliminary analyses (for rats Nos. 33 to 36) with the method for the hydroxy derivative (*10*) demonstrated that a substantial amount of this derivative [8] was present in both the bile and the urine.

Solubility behavior.—The partition between water and ether of N^{15} and of diazotizable material from bile and urine of rats fed AAF indicates that the material becomes more water-soluble during metabolism. Whereas unabsorbed AAF from the stomach of a rat is found entirely in the ether layer when an acetone extract is evaporated to dryness, taken up in ether and water and shaken repeatedly in a separatory funnel, only 10 to 25 percent of the total N^{15} of the urine from AAF-fed rats is found in the ether layer when the urine is shaken with ether. Over 90 percent of the ether-soluble N^{15} of the urine can be accounted for by the diazotization method, and a fraction of that which is found in the aqueous layer. Between 95 and 100 percent of the N^{15} and of the diazotizable material in the bile are found in the aqueous layer when bile is extracted with ether.

In feces about 25 percent of the total N^{15} appears to be water-extractable. If bile salts are added to the water the amount of extractable N^{15} may be increased to 60 percent of the total. If the feces are powdered and extracted with ether in an acid-aqueous suspension, 25 percent of the N^{15} is found in the acid-aqueous extracts and 10 percent in the ether. After repeated ether extractions of alkaline-aqueous suspensions of the feces, 55 percent of the N^{15} is found in the alkaline-aqueous layers and 24 percent in the ether. It is possible that part of the material in the feces is unabsorbed AAF or AF, while part consists of metabolites that have been excreted into the gastrointestinal tract by way of the bile. Preliminary solubility studies with a variety of organic solvents support the thesis that there are a number of metabolites of different solubilities present in both urine and feces.

Evidence for the presence of metabolites other than AF and 7-OH-AAF.—When the recoveries of diazotizable material in the bile and urine of rats Nos. 33 to 36 (table 4) were corrected for the lesser color production of the hydroxylated material [8] determined by the nitrite method, the corrected

[8] See footnote 8, page 95.

recoveries accounted for only 53 to 86 percent of the recoveries calculated from N^{15}. This observation has been made repeatedly with samples of urine from intact animals and will be reported in another paper. No enrichment of N^{15} was found in urea and creatinine isolated from samples of urine in which the diazotizable material was approximately one-third of that calculated from N^{15}. Moreover, the close parallelism in the urinary excretion of C^{14} from 2-AAF-9-C^{14} and the N^{15} from 2-AAF-N^{15} indicates that the nitrogen remains attached to the molecule.

The data reported here, together with solubility studies and the results from more recent analyses of urine for diazotizable material corrected for the hydroxy metabolites, are not conclusive, but they suggest the probable presence of other metabolites of AAF in addition to 7-OH-AF and 7-OH-AAF that contribute to the poor recoveries of diazotizable nitrogen.

Preliminary studies, using β-glucuronidase extracted from the livers and kidneys of mice, indicate that the glucuronides of AF or AAF, if present in the urine, give the same amount of color in the diazotization test before and after hydrolysis.

If the metabolism of AAF in the rat should resemble that of 2-acetamidonaphthalene, as reported by Manson and Young (*11*), other possible metabolites may be the sulfuric acid derivatives of AF and/or of AAF. It is reasonable to expect the hydroxylated derivative to be present in part as a glycuronide and as a sulfate. Following Manson and Young's procedure, however, we have not succeeded in isolating 2-aminofluorenyl-sulfuric acid from the urine of rats following the administration of AAF-N^{15}. One percent of the original N^{15} of the urine was found in the fraction expected to contain a sulfuric acid derivative, and half that amount could be accounted for by the diazotization method.

Comparison of the distribution and excretion of AAF with that of dimethylaminoazobenzene (DAB).—Whereas the carcinogenesis of DAB appears to be limited to the liver while that of AAF has been demonstrated in a great many types of tissue and primarily at sites distant from those of administration, it is interesting to find that there are in the rat many similarities in the distribution and excretion of AAF or its metabolites and DAB and/or its metabolites. Both compounds require multiple doses for the production of tumors and both are relatively insoluble in water. The azo nitrogens of DAB may be expected to have different metabolic fates than the amino nitrogen of AAF, yet both compounds may be considered as aromatic amines, with the DAB containing two additional potential aromatic amine groups in the azo linkage. The data reported by Berenbom and White (*12*) on the distribution in the liver and the excretion from Osborne-Mendel rats of the N^{15} of each of the three nitrogens of N^{15}-DAB closely resemble the observations on the fate of N^{15} of 2-AAF-N^{15} in the Buffalo rat. They found that approximately 70 percent of the administered N^{15} of DAB was excreted in the urine and 20 percent in the feces. Ninety percent or more of the N^{15} of DAB was excreted in 72 hours. The amount of DAB-nitrogen in the liver was very small, as was the AAF-nitrogen reported here. The excretion pattern of DAB-nitrogen, like that of AAF-nitrogen of the urine and feces of rats 8, 9 and 10, was not appreciably affected by continuous feeding of the carcinogens over a period of months. There are no data available to show whether or not DAB resembles AAF, during the first few hours following administration to the rat, in a varied but transient distribution to tissues other than the liver.

No reference has been found in the literature to the possible presence of DAB or its metabolites in bile, but Heidelberger and Weiss (*13*) have observed that excretion through the bile of rats and mice following intravenous injection is not unique, having been found with compounds of as varied chemical composition as inorganic colloids, stilbestrol, carcinogenic hydrocarbons, and steroid hormones.

Miller and Miller (*14*) have shown that DAB and other related carcinogenic dyes are bound by proteins of the liver. Following their procedure for the separation and washing of the material, it has been found in this laboratory that the N^{15} of 2-AAF-N^{15} is bound to protein fractions of the liver of rats and to a lesser degree to the protein fractions of several other tissues of the rat (*15*).

More knowledge of the mechanism of carcinogenesis is necessary before it can be inferred whether or not these similarities in the metabolic pathway and excretion patterns of DAB and AAF have any relation to their carcinogenic activity or are simply a result of the physical and chemical properties of the compounds.

Summary

1) A close similarity has been observed, qualitatively and quantitatively, between the distribution and excretion of N^{15} of 2-acetylaminofluorene-N^{15} (AAF-N^{15}) and that reported for C^{14} following the gastric or oral administration of 2-AAF-9-C^{14} to the rat, indicating that the molecule of AAF remains intact during metabolism so far as the nitrogen in position 2 and the carbon in position 9 are concerned.

2) After administration of AAF-N^{15} to the rat by gastric intubation, the N^{15} has a distribution in the tissues similar to that of the diazotizable nitrogen.

3) Recoveries of administered AAF by the diazotization method account for 29 to 54 (average 39) percent of the recoveries calculated from the N^{15} found in the excreta and the tissues, exclusive of the gastrointestinal tract. Approximately the same amounts of diazotizable nitrogen and N^{15} are found in the gastrointestinal tract when substantial amounts of AAF remain unabsorbed.

4) Following gastric intubation of AAF-N^{15} to rats, 56 to 71 (average 65) percent of the total amount of N^{15} recovered is found in the urine, and 29 to 44 (average 35) percent in the feces.

5) Failure in the extraction of a large portion of the N^{15}-containing material from feces for analysis by the diazotization method, and the interference by urinary pigment sometimes encountered with this method, account for some of the difference in the recoveries by diazotization and N^{15} methods.

6) The experimental results suggest that there may be present, in small amounts, other derivatives of AAF that, like the 7-hydroxy derivative, have lesser optical densities or may even fail to produce any color under the conditions of the diazotization method of analysis. This possibility has been discussed with regard to some of the probable metabolites.

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Tumors of the Glandular Stomach Induced in Mice of Six Strains by Intramural Injection of 20-Methylcholanthrene [1]

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The rarity of spontaneous gastric adenocarcinoma in animals has been repeatedly emphasized in the literature (*1–7*). The need for the development of a suitable experimental test object for the study of this important neoplasm in man is evident.

The present experiment was designed to test the effect of a single dose of a suspension of 20-methylcholanthrene and methocel injected into the wall of the glandular stomach of mice of six different strains.[5]

Literature

Waterman (*8*) obtained a "beginning" adenocarcinoma in one of six mice fed cholesterol oleate. The muscularis mucosae of the specimen was said not to be visible in the sections and therefore presumably was destroyed by the growth, but the muscularis propria was intact and there were no metastases. Some atypical structural characteristics of the gastric glands are revealed in illustrations 18 and 19 of Waterman's paper, but this evidence falls short of being indubitable proof that the lesion was an adenocarcinoma.

Domagk (*9*) described an experiment in which 20 mice were given two diets that were alternated each week and consisted of "ordinary corn feed" and of a mixture of rice and olive oil. Of the 20 mice, 9 were alive after one year. One animal was reported to have a gastric adenocarcinoma that extended to the serosa. There were no metastases. Illustrations 1 through 4 of Domagk's paper suggest that the lesion is indeed a genuine adenocarcinoma. Judging by the descriptions of the pathologic changes, the gastric lesions in the other 8 animals probably represented hyperplastic gastritis similar to that occurring spontaneously in several strains of mice (*10–12*).

A paper by Stewart and Lorenz (*13*) reported the induction of adenocarcinoma and other tumors of the glandular stomach of mice, following the intramural introduction of 20-methylcholanthrene, 1,2,5,6-dibenzanthracene, or of 3,4-benzpyrene dissolved in lard or mineral oil, suspended in horse serum, or impregnated on a cotton thread.

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[5] A preliminary report of the results of this experiment was presented at the Fourth Conference on Gastric Cancer at Stanford University in December, 1948 (*14*).

Materials and Methods

A total of 472 mice of strains A, BALB/c, C57BL, C3H, C3Hb, and DBA, 2 to 5½ months of age and approximately equally divided as to sex (233 males, 239 females) were used.

Under nembutal anesthesia administered intraperitoneally, the stomach was delivered through a midline incision of the epigastric region. Using a 1-cc. tuberculin syringe fitted with a 27-gauge needle, 0.01 cc. of a suspension (*1*) containing 0.3 mgm. of 20-methylcholanthrene was injected into the anterior wall of the gastric antrum.

Following withdrawal of the needle a small amount of the injected material occasionally leaked back along the needle track onto the serosa, which was wiped with absorbent cotton. A small yellow bleb in the tissue indicated a successful injection. The peritoneum and skin were closed by silk thread suture.

The methylcholanthrene-methocel suspension was not a permanently stable suspension and sedimentation was a constant hazard. Therefore, to insure the injection of a uniform amount of methylcholanthrene, the container was always shaken vigorously before loading the syringe and the latter was always rotated rapidly just prior to insertion of the needle into the wall of the stomach. An attempt was made to deposit the carcinogen in the submucosa, but this was not always successful; it was sometimes deposited in other sites in the wall of the stomach as, for example, in the muscularis propria and subserosa, and occasionally part or all of the material was accidentally injected into the lumen.

The animals were individually numbered, and caged in groups of from 5 to 10. The diet consisted of pellets of Purina dog chow with tap water *ad libitum*. The animals that had large palpable tumors and those ill from other causes were etherized and autopsied upon discovery of the condition. Of those found dead, the possible presence and degree of *post-mortem* change were always taken into consideration in the histologic evaluation of lesions. Mammary tumors were in some cases excised surgically from C3H female mice to give them a chance to live longer, and thereby to prolong the carcinogenic effect on the stomach. In extensive studies in this laboratory, metastases of mammary carcinoma to the stomach have not been observed. We feel certain that none of the lesions diagnosed as gastric carcinoma in this experiment were metastases from the neoplasms of the breast.

The stomach was always given first attention at autopsy. In some cases it was opened immediately, pinned to a stretching board, and fixed; in other cases the fixative was injected into it *in situ* after ligating the esophageal and duodenal ends. Freshly prepared Zenker's acetic fluid was used routinely to fix the tissue removed at autopsy, and in many instances a dilute solution of formaldehyde (USP 1:10) was also used. Tissue sections were prepared with hematoxylin and eosin, and by the periodic acid-Schiff reaction. Other methods, used as necessary, included van Gieson's connective tissue stain, various silver impregnation techniques for reticulum, and the phosphotungstic acid-hematoxylin stain.

Results and Comment

In diagnosing the gastric lesions listed in table 1, the terms "adenocarcinoma" and "adenoacanthoma" have been used to designate the malignant epithelial neoplasms that invaded all layers of the stomach and penetrated the serosa. The term "precancer" has been used in a broad sense to designate lesions (figs. 1 and 2) that suggested neoplasia and that varied in extent from atypical glands in the mucosa to a more advanced process with neoplastic invasion of the wall of the stomach but without penetration of the serosa. The term "sarcoma" has been used for the tumors apparently derived from the connective tissues. The mixed tumors have been classified as mixed adenocarcinoma and sarcoma, and as mixed adenoacanthoma and sarcoma. A study of the histologic classification of induced sarcomas of the stomach has been published (*15*).

Adenocarcinoma (figs. 3 to 7) of the glandular stomach occurred in 23 mice (4.9 percent); mixed adenocarcinoma and sarcoma in 46 mice (9.7 percent); adenoacanthoma (figs. 8 to 11) confined to the chamber of the glandular stomach in 12 mice (2.5 percent), in 5 of which there was also sarcoma (figs. 12, 13); a precancerous lesion in 221 mice (46.8 percent); and sarcoma in 39 mice (8.3 percent). Adenocarcinoma occurring either in pure form or as one of the components of a mixed tumor was found in 81 mice (17 percent). In the stomachs of 131 mice, listed in the last column of table 1, only foreign-body reaction or chronic inflammation existed, or no lesion was found. (Perhaps in some of the latter cases the lesion was missed.) However, the stomachs of 4 of the animals listed in the last column showed a squamous-cell carcinoma. One of these lesions of squamous-cell carcinoma was located chiefly in the forestomach and probably arose from the squamous epithelium of the mucosa of this chamber, although the glandular stomach was also involved by the tumor and hence its origin could not be stated positively. In another case, the lesion seemed to be confined within the limits of the glandular stomach, and very likely represented an adenoacanthoma with one-sided development. The other two growths were located principally in the pyloric portion of the stomach but involved both chambers, and hence in these cases it was impossible to determine the chamber in which the lesion was primary.

The tumors of the glandular stomach were usually evident at external examination or upon opening the stomach. Some of the small precancerous lesions were not observed at autopsy. The small lesions were more easily seen when the stomach was first fixed and then opened. These lesions were oval or round and their presence was suspected by the increased rigidity in the region of the injection site. The neoplasms were usually situated on the anterior wall of the antrum, at the injection site, but the larger ones were generally annular, and some of the largest growths extensively involved the stomach.

Many of the tumors, including the precancerous lesions, produced obstruction resulting in dilatation of the stomach. This was undoubtedly a contributing factor to the death of a number of animals. Only two gas-

TABLE 1.—*Lesions obtained in glandular stomach of mice treated with intramural injection of 20-methylcholanthrene*

Strain	Number and sex of mice used	Precancer	Adenocarcinoma	Adenoacanthoma	Mixed adenocarcinoma and sarcoma	Mixed adenoacanthoma and sarcoma	Sarcoma	No primary neoplastic lesion
BALB/c	M 25, F 34 = 59	12, 18 = 30	2, 1 = 3	0, 1 = 1	3, 6 = 9	1, 2 = 3	*3, 5 = 8	4, 1 = 5
C57BL	M 55, F 56 = 111	37, 45 = 82	1, 1 = 2	0, 1 = 1	3, 2 = 5	1, 0 = 1	0, 1 = 1	13, 6 = 19
C3H	M 46, F 45 = 91	18, 18 = 36	4, 4 = 8	1, 0 = 1	5, 8 = 13	1, 0 = 1	†4, 5 = 9	13, ‡10 = 23
C3Hb	M 42, F 31 = 73	18, 10 = 28	2, 3 = 5	1, 1 = 2	4, 4 = 8	0, 0 = 0	*7, 6 = 13	‡10, 7 = 17
DBA	M 29, F 39 = 68	1, 4 = 5	0, 0 = 0	0, 0 = 0	0, 1 = 1	0, 0 = 0	0, 2 = 2	28, 32 = 60
A	M 36, F 34 = 70	17, 23 = 40	3, 2 = 5	0, 2 = 2	6, 4 = 10	0, 0 = 0	5, 1 = 6	‡5, 2 = 7
Totals	472	221	23	7	46	5	39	131

* Includes one malignant hemangioendothelioma with metastases to liver.
† Including one osteogenic sarcoma.
‡ Squamous-cell carcinoma of forestomach.

tric adenocarcinomas showed invasion of adjacent organs and only one other metastasized.

Adenocarcinoma

The periods of time between the injection of methylcholanthrene into the wall of the stomach and the finding of adenocarcinoma at necropsy was as follows for the mice of the different strains: strain BALB/c—4, 13½, and 16 months; strain C57BL—4 and 5½ months; strain C3H—3, 7, 7, 17, 17½, 18, 20¼, and 22 months; strain C3Hb—6, 6½, 8½, 12½, and 13 months; strain A—5½, 13, 17½, 22, and 22 months.

The periods of time indicated represent the latent period of tumor induction plus the time the tumor-bearing animal survived until it died or was killed. The range was from 3 to 22 months. The occurrence of 7 carcinomas 3 to 6½ months following injection of the carcinogen indicates that the susceptibility of the glandular mucosa of the stomach to the effect of the carcinogen in these mice is not dissimilar to that of the skin and subcutaneous tissue.

Fourteen animals with adenocarcinoma died and nine were killed. One animal died immediately after the stomach was palpated and exhibited hemorrhagic ascites at autopsy. In two animals the tumor had extended into adjacent organs, into the pancreas in both cases, and into the omentum, gastrocolic and gastrosplenic ligaments in one. One additional animal had a metastasis in the lung. The primary tumor in this case measured 5 by 5 by 3 mm. and was in the anterior wall of the antrum close to the duodenum. Numerous other mice had larger primary tumors without showing metastasis.

Grossly, there was little to differentiate the precancerous lesion from the adenocarcinoma except the extent of the tumor. The adenocarcinomas were present as small nodules on the anterior wall of the antrum, or as thickened indurated lesions encircling the pylorus. Sometimes the lesion was visible as a thickened area on the mucosa of the stomach after it had been opened. This roughening of the mucosa or increase in the rigidity of the wall of the antrum was at times the only gross indication of a precancerous or small cancerous lesion. The tumors were gray in color except when they showed congestion, hemorrhage, necrosis, or secondary infection. Some puckering, particularly along the lesser curvature of the stomach, was noted occasionally due to retraction by the infiltrating tumor. Pronounced dilatation of the stomach due to pyloric obstruction was found rather commonly. Adhesions extending between the liver and the site of injection in the stomach were common. Acanthosis and hyperkeratosis of the forestomach and limiting ridge were sometimes evident.

Microscopically, there was moderate variation in the appearance of the glands composing the tumor and the cells lining them were frequently stratified, hyperchromatic, and relatively large with a high nucleo-cytoplasmic ratio. Many of the tumor cells were cuboidal and columnar in shape, and the cytoplasmic vacuoles seen in hematoxylin and eosin preparations were easily demonstrated to contain mucus by the use of

special stains. Occasionally neoplastic glands were lined with flattened epithelium. The undifferentiated cells were devoid of mucus, grew in nests and cords, and occasionally formed glands. None of the tumors was classified as the signet-ring-cell type of mucoid carcinoma. Mitotic figures were regularly present, but generally not numerous and not bizarre. The tumors infiltrated the muscularis propria in the form of cords and nests. Occasionally a slight fibroblastic reaction (desmoplasia) was seen in response to the epithelial downgrowth, but for the most part fibrous tissue, when present in appreciable amounts, was obviously a result of secondary inflammation. Hyalinization of the stroma of the tumors was uncommon. Degeneration of the blood vessels was frequently observed by the periodic acid-Schiff reaction.

Bone formation occurred in the stroma of two of the epithelial tumors. The same change has been described in induced gastric neoplasms in the rat and its possible pathogenesis has been discussed (*1*). The bony spicules were small, irregular in shape, and devoid of marrow elements. In a few mice a pink hyaline material was observed in the stroma of other epithelial tumors and was considered to be osteoid in nature. One mouse developed an osteogenic sarcoma and this type of lesion has also been induced in the rat (*1*). Twelve malignant mixed tumors having squamous-cell carcinoma as one of the components arose in the glandular stomach. Under the experimental conditions a change from gastric glandular to squamous epithelium occurs much more frequently in gastric cancer in the mouse than it does in man under the circumstances that induce human gastric cancer. The rat (*1*) responds like the mouse to the development of squamous-cell metaplasia in the glandular mucosa following the injection of methylcholanthrene into the wall of the stomach.

Adenomatous polyps occurred in the pylorus in 4 mice representing 3 different strains (A, C57BL, and BALB/c). The glands composing the polyps were identical with those found in the invasive neoplasms, and have therefore been classified as precancerous. It should be noted that in this and other experiments, adenomatous polyps in mice do not usually precede the development of gastric carcinoma induced by methylcholanthrene. The same is true of induced carcinoma of the small intestine of mice (*16*).

Other changes occasionally observed in some of the mice were emaciation, bronchiectasis, pneumonia, primary pulmonary tumor, pericarditis, mediastinitis, dilatation of the uterus, sarcosporidiosis of the myocardium, pigmentation of liver and spleen, focal necrosis of liver, focal glossitis, Klossiella-Muris infection of kidney, atrophy of testis, visceral amyloidosis, nephritis, a hemangioma of liver (1 case), mammary carcinoma (2 cases), carcinoma of ovary (1 case), and a primary hemangioendothelioma of the chest wall (1 case).

Sarcoma

Sarcoma occurred in 39 animals, and mixed sarcoma and carcinoma in 51 others, making a total of 90 animals that developed sarcoma. No strain of mouse failed to develop sarcoma, although mice of strains C57BL and of DBA were relatively resistant to the induction of this lesion.

The histologic types of induced sarcomas that may occur in the stomach have been described (*15*). In 51 cases, the sarcoma was mixed with atypical epithelial elements that appeared neoplastic and that were diagnosed as carcinomatous elements mixed with the sarcoma. Since both the glandular mucosa and the supporting mesenchymal tissues of the stomach were exposed to the carcinogen in this experiment, it was not surprising that sarcoma developed so frequently. There was no suggestion of gradation between the cell types of the mixed tumors, and hence it is believed that these neoplasms were actually examples of mixed primary malignant tumors.

Transplantation

Transplantation to genetically compatible hosts was attempted in 50 cases. The following tumors were successfully transplanted: 1 adenocarcinoma; 4 adenoacanthomas, all of which grew as squamous-cell carcinomas in the transplants; 1 squamous-cell carcinoma; 15 sarcomas; and 1 mixed adenoacanthoma-sarcoma, the transplants of which grew as sarcoma. One precancerous lesion, 2 adenoacanthomas, 1 squamous-cell carcinoma, 3 sarcomas, and 6 mixed adenoacanthoma-sarcomas failed to grow upon transplantation.

Discussion

Twenty-three mice, representing 4.9 percent of the animals used, developed unmixed adenocarcinoma of the glandular stomach in this experiment. However, 81 mice (17 percent of the animals treated) developed adenocarcinoma, either in pure form or as one of the components of a mixed tumor (*viz.* adenoacanthoma, mixed adenocarcinoma-sarcoma, or mixed adenoacanthoma and sarcoma). In addition, it is possible that some of the lesions classified as precancerous were in reality carcinoma, since the criteria used for distinguishing these conditions were unusually rigid. Even so, it is evident that although the present technique for inducing gastric adenocarcinoma is the best available at the present time, the method leaves much to be desired. It would be advantageous to have a technique for inducing this lesion in nearly 100 percent of mice under experimentation, and it would be an added advantage if the resulting tumors spread and metastasized more aggressively so as to simulate more closely the biologic behavior of the corresponding lesion in man.

As long as no method exists for inducing gastric adenocarcinoma except by introducing a carcinogen into the wall of the viscus, and unless or until a carcinogen specific for epithelium is developed, sarcoma will almost certainly occur as a complication of the experimental procedure. By this method, both the mucosa and the connective tissue elements of the stomach are exposed to the effects of the carcinogen. There is at present no known carcinogen that has a purely selective effect on the epithelial elements of the gastric mucosa. The mesenchymal tissues of the stomach are also susceptible to such carcinogens as have been tested. All attempts to induce gastric adenocarcinoma by oral administration of carcinogen

have been unsuccessful (*17*). There are carcinogens that induce cancer in tissues distant from the site of inoculation, such as acetylaminofluorene, and it is not improbable that a method may be developed for induction of gastric adenocarcinoma by injecting the carcinogen at a site remote from the stomach.

The occurrence of primary adenoacanthoma in the gastric antrum of twelve mice (5 of these lesions were mixed with sarcoma) is in contrast to the rare occurrence of spontaneous neoplasms of this type in man. All of these lesions in mice proved to arise from the gastric glandular mucosa and not from the squamous-cell mucous membrane of the forestomach. The rat (*1*) is also likely to exhibit squamous-cell neoplastic elements in tumors induced at the antrum by the intramural injection of 20-methylcholanthrene. In the present experiment, there was one squamous-cell carcinoma that was not mixed with glandular elements and that appeared to be confined to the chamber of the glandular stomach. Although absolute proof is lacking, it is believed that this tumor arose as an adenoacanthoma and that the squamous-cell carcinoma represented a one-sided development. Three other squamous-cell carcinomas were observed in this experiment. Two of these were located in the region of the limiting ridge and involved both chambers, and thus could have arisen from the mucosa of the forestomach. The third was confined to the forestomach and so was primary there. The carcinogen may work its way into the submucosa of the forestomach or may come in contact with the wall of the forestomach at the time of injection, thus inducing neoplasms of the forestomach composed of squamous cells.

The absence of neoplastic lesions of the glandular stomach in 131 mice is accounted for chiefly by the refractoriness of this organ to the carcinogenic effects of methylcholanthrene in strain DBA mice. Even so, from 8 percent to 25 percent of the other five strains of mice utilized exhibited no tumor of the glandular stomach at necropsy. There are a number of possibilities that might account for this. It is not surprising to find some animals in an experiment resistant to the induction of cancer and this is a reasonable assumption in the present experiment. Other mice died early in the experiment before the carcinogenic effects of the methylcholanthrene became manifest. The carcinogen may have been injected in low doses due to sedimentation, or injected into the lumen of the stomach, or into the lamina propria from which site it was discharged into the lumen following ulceration. There may have been failures to detect lesions at necropsy, or errors in trimming or blocking the sections. However, the DBA strain of mouse is peculiarly refractory to the induction of all types of malignant neoplasms of the stomach by the present technique. Despite the fact that these mice spontaneously develop a hyperplastic gastritis that resembles the gastric lesion of the strain I mouse, only 3 malignant tumors, one of which contained epithelial elements, developed in these mice. Precancerous lesions were diagnosed in 5 mice of the DBA strain, but these may be open to question because of the difficulty of distinguishing between an induced precancerous lesion

and the spontaneous hyperplastic process in the glandular mucosa of the stomach in DBA mice. On the other hand, a precancerous lesion may have been disregarded in some instances for the same reason. Since only one genuinely malignant epithelial type of neoplasm occurred in the strain DBA mice, it is justifiable to require that very careful consideration be given to all the evidence before a lesion is diagnosed as precancerous in mice of this strain.

No great difference has been found with respect to the neoplastic response of the gastric glandular mucosa in the other five strains of mice, except that the C57BL mice seemed to respond more slowly. Sarcoma of the stomach also developed less frequently in mice of the C57BL strain, which is compatible with the low yield of sarcoma obtained when the subcutaneous tissues of this animal are exposed to methylcholanthrene.

A final point that needs emphasis is the difference in the morphology of the lesions that develop at the antrum in the mouse and the rat (*1*) following the intramural injection of methylcholanthrene. The rat frequently develops an adenomatous diverticulum which appears to be unique for this species.

Summary and Conclusions

1) A total of 472 mice of six different strains, approximately equally divided as to sex, received an injection of 20-methylcholanthrene into the wall of the stomach. The animals were allowed to live out their lives or were killed when death appeared to be imminent.

2) The following lesions of the glandular stomach were found: precancerous lesions 221; adenocarcinoma 23; adenoacanthoma 7; mixed adenocarcinoma and sarcoma 46; mixed adenoacanthoma and sarcoma 5; sarcoma 39.

3) The DBA strain of mouse was refractory to the induction of all types of malignant neoplasms of the stomach by the present technique.

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PLATE 5

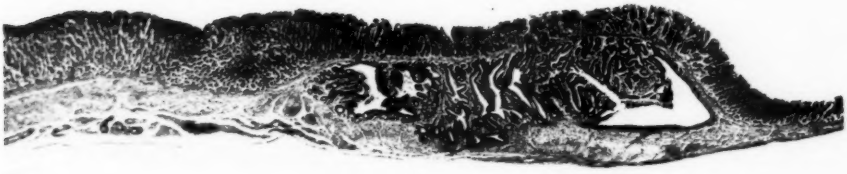
Lesions in glandular stomach of mice injected intramurally with 0.01 cc. of a suspension containing 0.3 mgm. of 20-methylcholanthrene. Hematoxylin and eosin.

FIGURE 1.—Precancerous lesion. The plane of the section passes through the mid-portion of the lesion and shows the neoplastic proliferation of glands extending beneath the muscularis mucosae and infiltrating the muscularis propria. × 30

FIGURE 2.—Precancerous lesion. The plane of the section is somewhat lateral to the mid-portion of the lesion. The neoplastic glands infiltrate the submucosa and muscularis propria. × 25

FIGURE 3.—Adenocarcinoma. The neoplasm has penetrated all coats of the glandular stomach. × 27

1



2

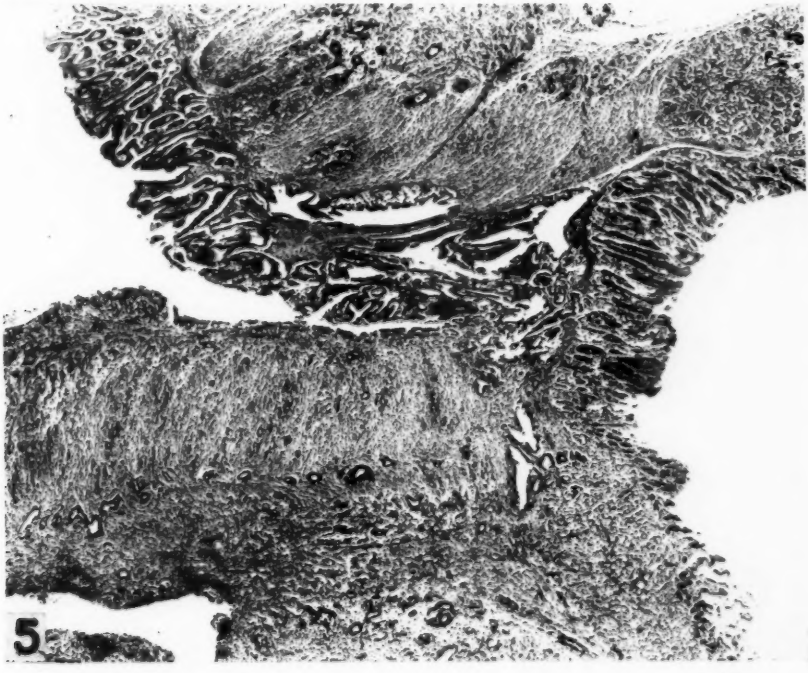
3

255905—53——17

PLATE 6

FIGURE 4.—Adenocarcinoma of the glandular stomach of a mouse injected intramurally with 20-methylcholanthrene. The lumen of the stomach which is markedly dilated is on the left, and the lumen of the duodenum on the right. More of the details of this lesion are shown in figures 5, 6, and 7. Hematoxylin and eosin. × 15

FIGURE 5.—Higher magnification of the lesion in the antrum shown in figure 4. The mucous surface is ulcerated on one side and shows piling up of the glands on the other side. The muscularis propria and the serosa are infiltrated by the adenocarcinoma. The continuity of the gastroduodenal lumen is not shown in the plane of the section. × 37

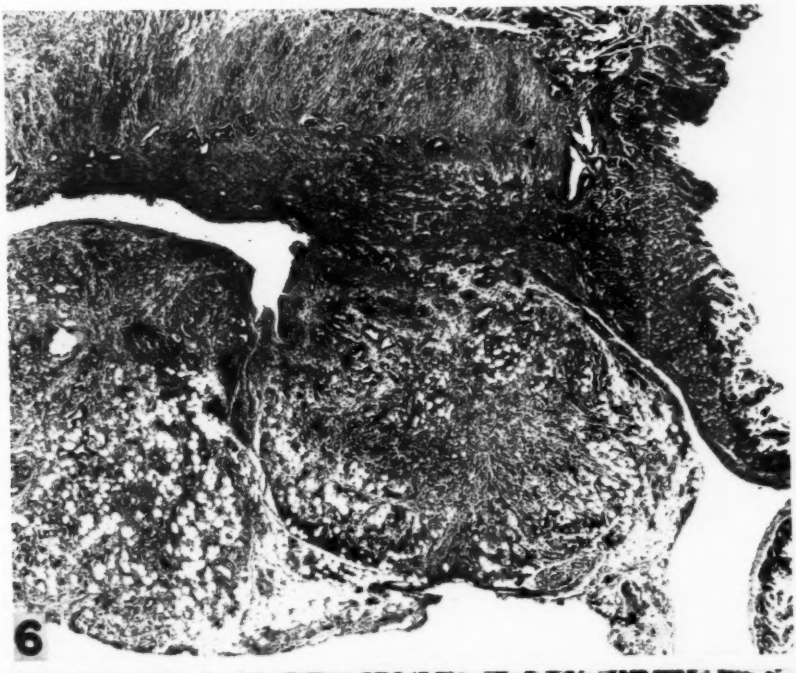


255905—53——18

PLATE 7

FIGURE 6.—Higher power of figure 4 showing the wall of the antrum and attached mesenteric fat, infiltrated by adenocarcinoma. × 33

FIGURE 7.—Showing the appearance of the acinous structures composing the adenocarcinoma of figures 4, 5, and 6. × 270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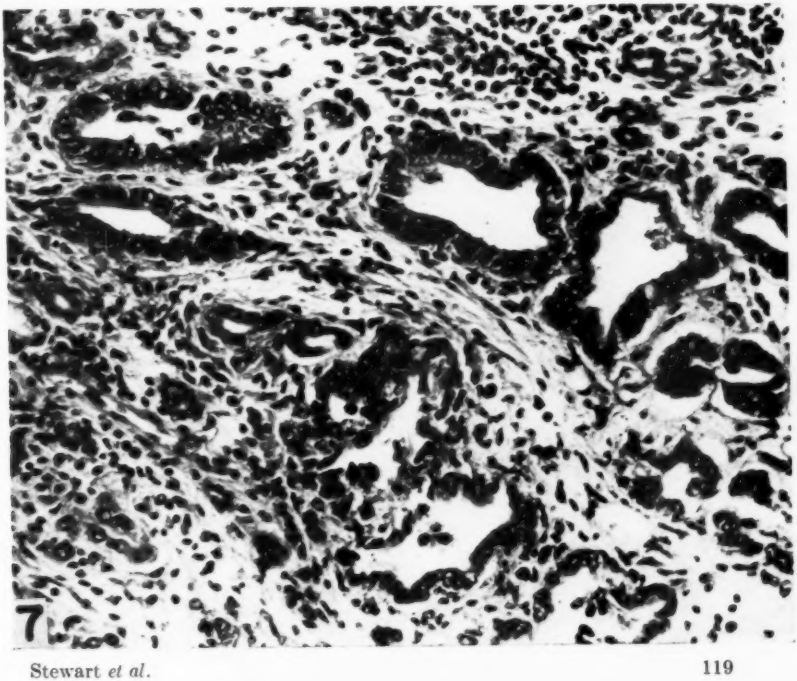
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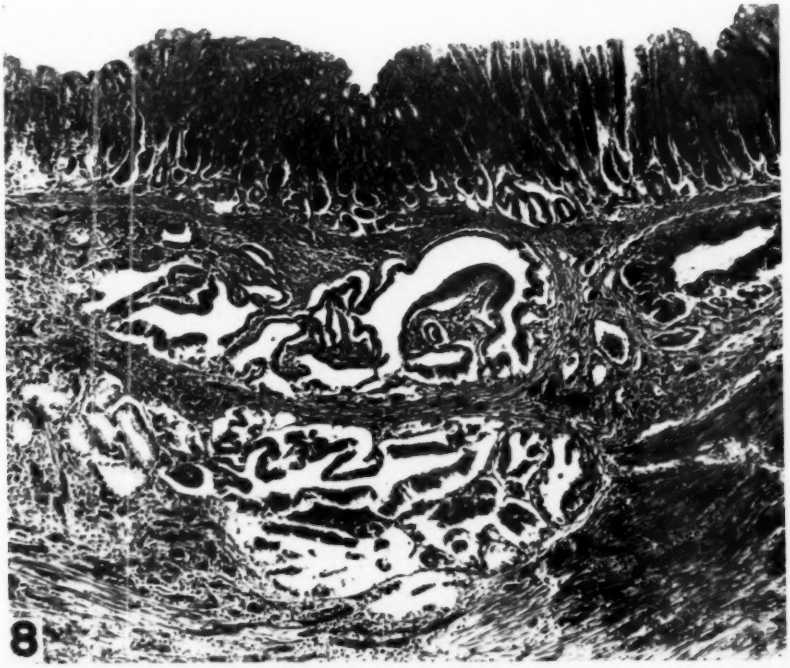
PLATE 8

FIGURE 8.—Adenoacanthoma in glandular stomach of mouse injected intramurally with 20-methylcholanthrene, showing the glandular elements that had evidently proliferated independently. Hematoxylin and eosin. × 130

FIGURE 9.—Adenoacanthoma in glandular stomach of mouse injected intramurally with 20-methylcholanthrene. This illustration shows that the squamous-cell neoplastic elements had evidently proliferated independently of the glandular elements shown in figure 8. Hematoxylin and eosin. × 480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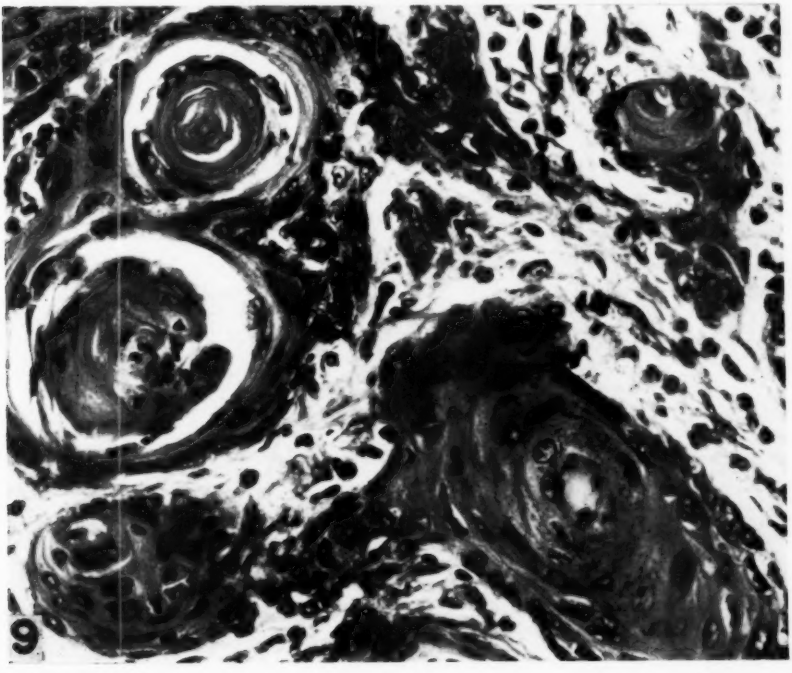
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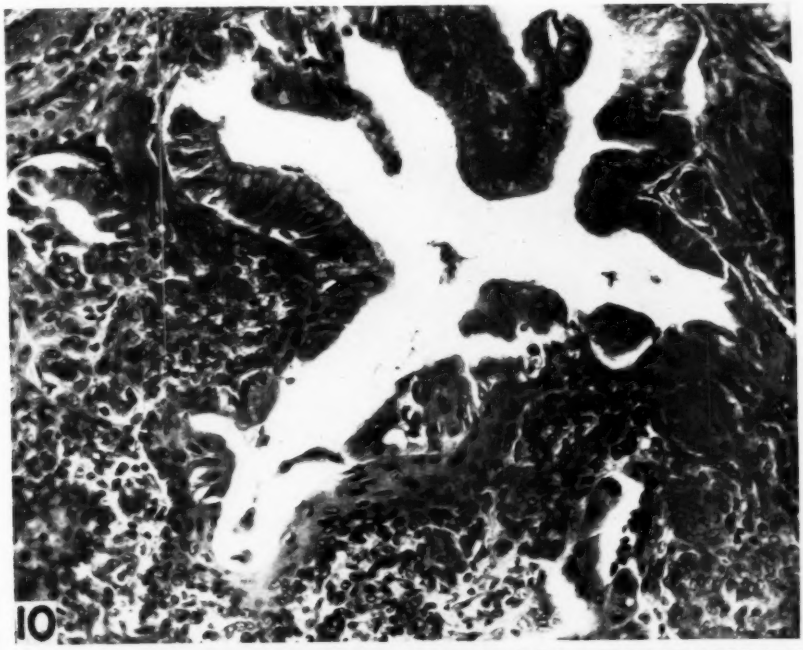
PLATE 9

FIGURE 10.—Adenoacanthoma of glandular stomach of mouse injected intramurally with 20-methylcholanthrene, showing acinar structure lined in part by squamous cells and in part by glandular cells. Hematoxylin and eosin. × 250

FIGURE 11.—Adenoacanthoma of glandular stomach of mouse injected intramurally with 20-methylcholanthrene, showing a microscopic field in which both squamous and glandular cells line an acinar structure. Hematoxylin and eosin. × 150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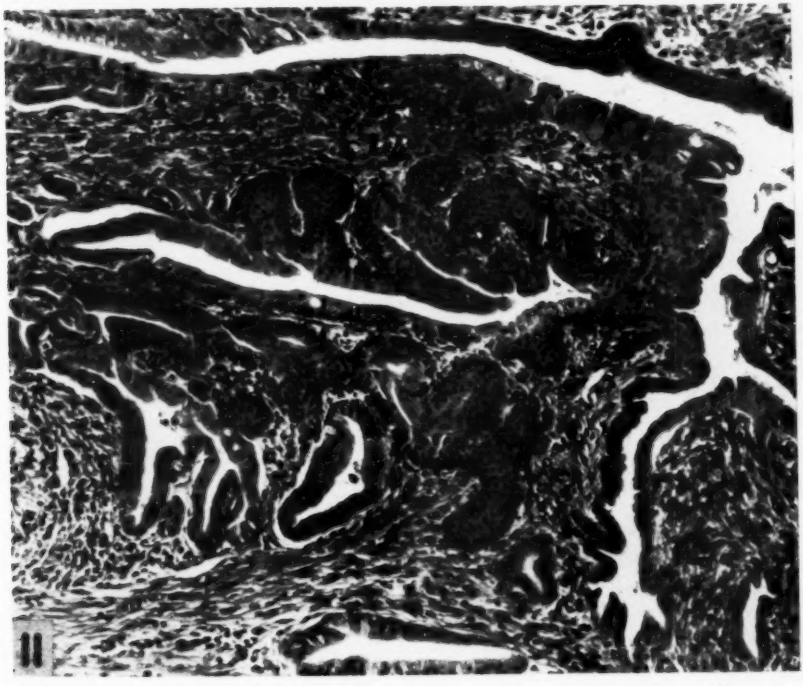
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PLATE 10

FIGURE 12.—Mixed adenoacanthoma and sarcoma primary at the limiting ridge of the stomach of a mouse injected intramurally with 20-methylcholanthrene. The wall of the glandular stomach is shown on the right and the wall of the forestomach on the left. This is one of the few lesions that was polypoid. Most of the squamous-cell elements are in that portion of the nodule nearest the glandular stomach. Hematoxylin and eosin. × 15

FIGURE 13.—Mixed adenoacanthoma and sarcoma of the glandular stomach of a mouse injected intramurally with 20-methylcholanthrene, showing all three elements in this section. Hematoxylin and eosin. × 110

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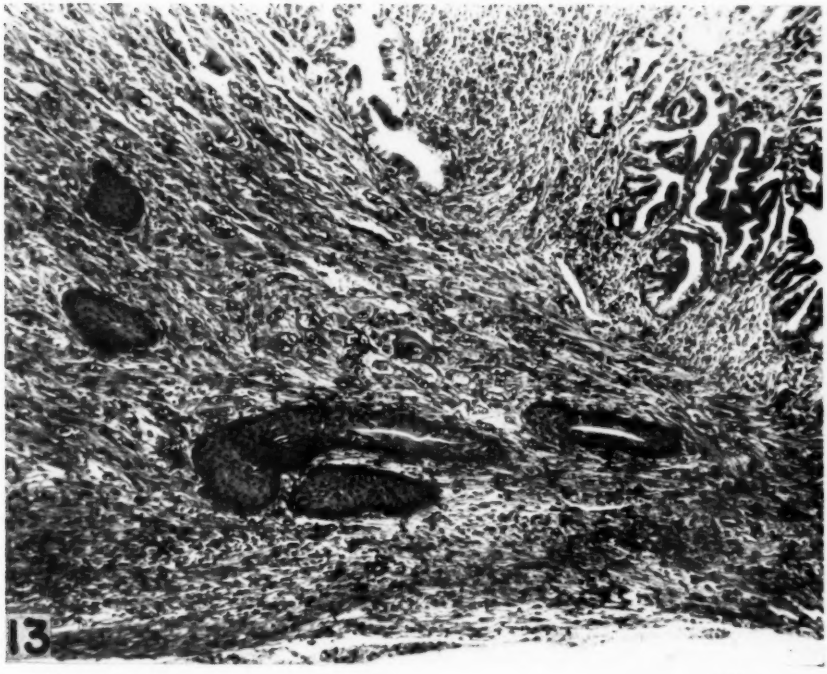

13

Occurrence of Pulmonary Tumors in Mice of Strains A, BL, and SWR and Their F_1 Hybrids Following Intravenous Injection of 1,2,5,6-Dibenzanthracene[1,2]

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A study was made of the incidence of induced pulmonary tumors in hybrid mice resulting from crosses between three relatively high-pulmonary-tumor strains. These were strains A, SWR (Swiss), and BL (Bagg L). Heston (*1*) has observed an incidence of 90 percent for spontaneous pulmonary tumors in strain A mice killed at 18 months of age. Lynch (*2, 3*) has recorded incidences of pulmonary tumors of 80 percent for strain SWR mice over 18 months of age (46.1 percent for all ages) and of 37 percent for strain BL mice over 12 months of age (26.3 percent for all ages). The usual procedure of crossing high- and low-tumor strains has not been followed in the hybrid crosses produced. Instead, crosses have been made between the three high-tumor strains for the purpose of ascertaining whether the hybrids would be comparable to either strain, would be intermediate between the two strains, or would respond more than the higher strain or less than the lower strain owing to a combination of factors for susceptibility or resistance.

Experimental Procedure

The strain A, strain SWR, and strain BL mice used in this experiment were from the colonies of inbred mice maintained by the authors at the National Cancer Institute. The SWR and BL strains were obtained in June 1951 from Dr. Clara J. Lynch of the Rockefeller Institute for Medical Research. Strain SWR has been inbred for 44 generations and strain BL for 49. Strain SWR differs in this respect from the Swiss mice in widespread use.

Approximately 50 animals (distributed equally between the sexes) of each of the three strains and of (A × BL)F_1, (A × SWR)F_1, and (SWR × BL)F_1 hybrids were injected intravenously at 2 months of age with 0.5 mg. of 1,2,5,6-dibenzanthracene dispersed in 0.5 cc. of horse serum.[4] The animals were housed in plastic cages, 8 animals to a cage, and were

[1] Received for publication February 27, 1953.
[2] Presented at the meeting of the American Association for Cancer Research, Chicago, Illinois, April 9-11, 1953.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[4] The dispersion used in this experiment was prepared by Dr. Egon Lorenz of the National Cancer Institute.

constantly supplied with Derwood food pellets and tap water. The animals were autopsied when 6 months of age. Nodules present on the surface of the lung were counted with the aid of a dissecting microscope, and the number recorded.

Results and Discussion

All the animals in this experiment developed pulmonary tumors. For this reason differences could not be detected from incidence data. However, susceptibility to induced pulmonary tumors could be measured by the number of tumor nodules per individual. In previous experiments (*1, 2, 4, 5*) this method has been used as a measure of susceptibility and as a means of determining differences in susceptibility of various groups. The average number of nodules for the males and females of each of the three strains and of the three F_1 hybrids are shown in table 1. The average number of nodules in the BL strain was 25.5 for males, 16.5 for females; in the A strain, 65.4 for males, 66.8 for females; and in the SWR strain, 106.8 for males and 116.2 for females. The average number of nodules was higher in the males than in the females in strain BL but higher in the females in strains SWR and A. However, the difference was highly significant only in strain BL ($t=3.41$, $P<.01$) but not significant in strain A ($t=0.30$, P is between .8 and .7) or in strain SWR ($t=1.43$, P is between .2 and .1). It is interesting to note that the strain SWR mice, which have been shown to have a lower incidence of spontaneous pulmonary tumors than strain A mice, had a higher average number of induced nodules. Lynch (*2*), who injected strain SWR and strain BL mice with 0.125 mg. of dibenzanthracene at 2 to 4 days of age, found tumor nodules ranging in number from 24 to 86 in SWR animals at 2 to 2½ months of age and as many as 150 nodules at 6 to 7 months of age. She also found nodules ranging in number from 27 to 61 at 6 to 7 months of age in strain BL mice. The average number of nodules for the (SWR × BL)F_1 hybrids was 38.6 for males and 29.8 for females, a response intermediate between those of the two parent strains. This same situation existed in the (A × BL)F_1 hybrids and in the (A × SWR)F_1 hybrids where the average number of nodules was 61.8 for males and 40.5 for females in the former, and 84.6 for males and 75.0 for females in the latter case. The average number of nodules in the hybrids was higher in the males than in the females. The difference was highly significant in the (SWR × BL)F_1 hybrids ($t=3.03$, $P<.01$) and also in the (A × BL)F_1 hybrids ($t=6.09$, $P<.01$), but of borderline significance in the (A × SWR)F_1 hybrids ($t=2.03$, P is between .05 and .02). The (SWR × BL)F_1 hybrids showed a lower response to the induction of pulmonary tumors than did the (A × BL)F_1 hybrids. When susceptibility was measured by the average number of nodules, strain SWR showed a higher degree of susceptibility than strain A, but when measured by breeding tests (outcrossing to strain BL) strain A demonstrated a higher degree of genetic susceptibility. This result was more in line with observations on spontaneous incidences of pulmonary

tumors in which strain A has been shown to have a higher incidence than strain SWR.

TABLE 1.—*Occurrence of induced pulmonary tumors in three inbred strains and their F_1 hybrids*

Strain or hybrid	Males			Females		
	No. of animals	Average no. of nodules	Range	No. of animals	Average no. of nodules	Range
BL (Bagg L)	25	25.5	7–46	28	16.5	2–33
A	25	65.4	23–94	26	66.8	43–99
SWR (Swiss)	25	106.8	77–156	22	116.2	63–159
(SWR × BL)F_1	30	38.6	13–55	27	29.8	14–65
(A × BL)F_1	25	61.8	35–76	27	40.5	17–92
(A × SWR)F_1	28	84.6	43–111	28	75.0	40–117

The degree of susceptibility of the F_1 hybrids never fell beyond the limits of the parent strains as it might have been expected to do through a combination of genes giving an additive effect. Inheritance of susceptibility to induced pulmonary tumors, as in spontaneous pulmonary tumors, has been shown to be on a multiple-factor basis with cumulative effect of the factors (*4*). The results obtained in this experiment could be expected from various combinations of multiple genetic factors, some of which were dominant and some recessive.

Summary

The three high-pulmonary-tumor strains of mice, A, SWR, and BL and their F_1 hybrids were tested for susceptibility to pulmonary tumors induced with 1,2,5,6-dibenzanthracene.

Pulmonary tumors occurred in all animals but the groups varied in susceptibility as measured by the average number of nodules per mouse.

The susceptibility of each of the three hybrid groups, (SWR × BL)F_1, (A × BL)F_1, and (A × SWR)F_1, was intermediate between those of their respective parent strains and never fell beyond the limits of the parent strains.

Although strain A has a higher incidence of spontaneous pulmonary tumors than does strain SWR, the degree of susceptibility of strain A to induced pulmonary tumors as measured by the average number of nodules induced was less than that of strain SWR. However, (SWR × BL)F_1 hybrids showed a weaker response to the induction of pulmonary tumors than did the (A × BL)F_1 hybrids. Thus, the ability of these two strains to transmit susceptibility to their respective offspring was more in

line with their degree of susceptibility as measured by incidence of pulmonary tumors than their susceptibility as measured by average number of induced nodules.

The males had on the average more tumors than did the females in all groups except the SWR and A. The sex difference was significant at a less than one-percent level in the BL, the (SWR $\times$ BL)F_1, and the (A $\times$ BL)F_1 groups.

References

(*1*) Heston, W. E.: Inheritance of susceptibility to spontaneous pulmonary tumors in mice. J. Nat. Cancer Inst. 3: 79–82, 1942.

(*2*) Lynch, C. J.: Lung tumors following intraperitoneal injection of 1:2:5:6-dibenzanthracene into young mice of three strains. Proc. Soc. Exper. Biol. & Med. 52: 368–371, 1943.

(*3*) ———: Studies on the relation between tumor susceptibility and heredity. III. Spontaneous tumors of the lung in mice. J. Exper. Med. 43: 339–355, 1926.

(*4*) Heston, W. E.: Genetic analysis of susceptibility to induced pulmonary tumors in mice. J. Nat. Cancer Inst. 3: 69–78, 1942.

(*5*) Lynch, C. J.: Influence of heredity and environment upon number of tumor nodules occurring in lungs of mice. Proc. Soc. Exper. Biol. & Med. 43: 186–189, 1940.

Occurrence of Tumors in Mice Injected Subcutaneously with Sulfur Mustard and Nitrogen Mustard [1]

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The demonstration of mutagenic action of the mustards (*1*) has recently aroused considerable interest in testing them for carcinogenic capacities. In this laboratory it has been shown (*2, 3*) that both sulfur mustard, bis(2-chloroethyl) sulfide, and the nitrogen mustard, methyl bis(2-chloroethyl) amine hydrochloride, which are strongly mutagenic when tested on *Drosophila* and other lower organisms, were potent in the induction of pulmonary tumors when injected intravenously in strain A mice. On the other hand mustard oil, allyl isothiocyanate, which was a weak mutagen, did not increase the occurrence of pulmonary tumors in strain A mice.

It is recognized that the induction of pulmonary tumors by the intravenous injection of a compound into mice of the genetically highly susceptible strain A constitutes one of the most delicate tests available for carcinogenic action. It is well, however, to supplement this test by those in which other routes of application of the agent and other strains of mice are employed. It has recently been shown that mustard gas, when inhaled by strain A mice, significantly increased the incidence and average number of pulmonary tumors in the animals (*4*). The present paper reports the induction of tumors in strains A, C3H, and C3Hf mice at the site of subcutaneous injection of both sulfur mustard and the nitrogen mustard, methyl bis.

Materials and Methods

The strain A mice used in this study were from the colony of the highly inbred strain A that has been maintained in this laboratory and used extensively for studies of pulmonary tumors. The highly inbred strain C3H used is particularly well known as a high-mammary-tumor strain, but the strain is also susceptible to subcutaneous tumors induced by the polycyclic hydrocarbon carcinogens. The present subline was derived from animals received from Dr. H. B. Andervont in 1941. Strain C3Hf is like C3H except that it does not carry the mammary tumor agent and,

[1] Received for publication March 10, 1953.

[2] With the technical assistance of W. D. Levillain.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

thus, has a lower incidence of mammary tumors than that observed in C3H. The strain C3Hf was developed in this laboratory from a litter removed from a strain C3H mother by cesarean section and foster-nursed upon a strain C57BL female in 1945. Strain C3Hf has been previously reported as C3Hb.

The two compounds tested were sulfur mustard, bis(2-chloroethyl) sulfide,[4] and the nitrogen mustard, methyl bis(2-chloroethyl) amine hydrochloride.[5]

There were nine experiments in the study. All injections were made subcutaneously in the mid-dorsal region. The amount injected and the number of injections together with the number and strain of mice used in each experiment are shown in table 1. In certain experiments some of the animals died from the immediate effect of the mustard injected, or died at a later date and no record was obtained. In these instances the number injected along with the effective total is indicated.

Experiments I and II were started on September 19, 1949. Experiment I was to test the action of sulfur mustard and experiment II was to test that of the nitrogen mustard. Strain C3H males and females were used in each experiment. Since the vehicle for the sulfur mustard was olive oil the controls of experiment I were injected with olive oil, but since the nitrogen mustard in experiment II was injected in water it was not considered necessary to inject the controls in this experiment.

At the end of the first week it was apparent that the animals that had received the .1 cc. of a .1 percent solution of sulfur mustard would not be able to tolerate more, so the injection was not repeated. During the next few days half of the animals died. The injection of nitrogen mustard in experiment II was repeated at weekly intervals for a total of 8 injections, when they were discontinued because ulcers had appeared in most of the animals at the site of injection.

The injections for experiments III, IV, and V were started on October 3, 1949. Experiment III was a repeat of the test of sulfur mustard in experiment I except that the dosage was reduced to .05 cc. of a .05 percent solution so that repeated injections could be tolerated. The injections were repeated at weekly intervals for a total of 6 injections, when they were discontinued because of ulcerations in most of the animals. Controls of experiment I served as controls of experiment III.

Experiment IV was likewise a test of sulfur mustard but using strain C3Hf females to avoid the spontaneous mammary tumors that could be expected in C3H females. The injections were repeated in this experiment as in experiment III. A large number of virgin C3Hf females were being observed concurrently for incidence of spontaneous tumors in another study (*5*) and could thus satisfy the needs for controls in this experiment.

Experiment V was a test of sulfur mustard injected into strain A mice. The injections were done as in experiments III and IV except that they

[4] Supplied by the Medical Division, Army Chemical Center, Md.
[5] Supplied by Merck and Company, Inc., Rahway, N. J.

were discontinued after the fifth injection instead of the sixth because of ulceration and general poor condition of the injected mice. The controls of this experiment received 5 weekly injections of olive oil.

From the observations of the first five experiments during the first year it was found that tumors could be induced at the site of the subcutaneous injection with either the nitrogen or sulfur mustard, but it appeared that the final incidence would probably be low and, thus, observation on a greater number of animals was desirable. Accordingly, experiments VI, VII, VIII, and IX were started November 13, 1950. Since it appeared that strain C3H mice were more susceptible than strain A mice to the induction of tumors at the site of the subcutaneous injection of these compounds, only C3H and C3Hf mice were used in these last four experiments. Furthermore, only males were used. In experiments VI and VII sulfur and nitrogen mustard respectively were tested in strain C3H mice, and in experiments VIII and IX the two compounds respectively were tested in strain C3Hf mice. In each of these experiments injections were done at two-day intervals for a total of 6 injections, by which time ulcers were beginning to appear. Since no tumors had appeared at the site of the injection of olive oil in the former controls, the controls of these experiments were not injected. Controls listed in table 1 under experiment VII also served for experiment VI, and those listed under experiment IX also served for experiment VIII.

Following the injections the animals were kept under observation for tumor formation. Throughout the experiments all mice were kept in plastic cages with 8 mice to a cage and were given an unlimited supply of Derwood food pellets and tap water. Autopsies were done on the animals after tumors were found or when the animals were found to be moribund or had died in the cages. The tumors were routinely fixed in Fekete's modification of Tellyesniczky's fluid (70 percent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part). They were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results

Tumors occurring in the experimental animals at the site of injection of the mustards and at sites remote from the site of injections are listed in table 1 along with the tumors occurring in the control mice.[5] It is particularly significant that in 20 of the animals injected with the mustards tumors appeared at the site of injection, whereas in none of the controls injected with olive oil did tumors appear at the site of injection and in none of the uninjected controls did tumors appear in the mid-dorsal region. Predominant among the tumors at the site of injection were subcutaneous tumors composed of fusiform cells, which were diagnosed as sarcomas. No special studies were made in an attempt to identify these tumors more exactly, but they appeared to be derived from fibroblasts. Five of these were in strains C3H and C3Hf mice injected with

[5] The author gratefully acknowledges the assistance of Dr. Thelma B. Dunn, who examined the slides and concurred in the diagnoses.

TABLE 1.—*Occurrence of tumors in mice injected subcutaneously with sulfur mustard (HS) and nitrogen mustard (HN_2)*

Experiment No.	Injection	No. of inj.	Mice injected					Tumors at site of injection			Tumors at other sites		
			Strain	Sex	Number	Age at start of exp. (*mos.*)	Avg.* mos. survival	No. mice with tumor	Diagnosis	Avg.* mos.	No. mice with tumor	Diagnosis	Avg.* mos.
I	.1 cc. .1% soln. HS in olive oil	1	C3H...	♂	(8) 5	4	6	0	.....	.....	0	.....	.....
				♀	(8) 3	4	6.3	0	.....	.....	3	Mammary tumor..	6.3
	.1 cc. olive oil.....	1	C3H...	♂	8	4	14	0	.....	.....	1	Pulmonary tumor.	17
											2	Hepatoma.....	17
				♀	8	4	7.4	0	.....	.....	7	Mammary tumor..	7.7
II	.025 mg. HN_2 in .25 cc. H_2O.	8	C3H...	♂	(8) 7	3–4	10.6	1	Papilloma.....	9	3	Pulmonary tumor.	13.3
								1	Squamous-cell carcinoma.	13			
				♀	8	3–4	8.6	1	Papilloma.....	8	2	Pulmonary tumor.	13
											7	Mammary tumor..	7.9
	Not injected.....	....	C3H...	♂	(8) 6	3–4	14.3	.....	.....	.....	1	Pulmonary tumor.	17
				♀	8	3–4	11.1	.....	.....	.....	2	Pulmonary tumor.	10
											7	Mammary tumor..	10.4
III	.05 cc. .05% soln. HS in olive oil.	6	C3H...	♂	8	3	14.3	1	Sarcoma.....	10	2	Pulmonary tumor.	15.5
				♀	8	3	8.9	0	.....	.....	8	Mammary tumor..	8.9
											1	Hepatoma.....	10
IV	.05 cc. .05% soln. HS in olive oil.	6	C3Hf..	♀	(10) 9	2	15.4	2	Sarcoma.....	12.5	1	Pulmonary tumor.	18
								1	Mammary tumor.	14	1	Mammary tumor..	18
V	.05 cc. .05% soln. HS in olive oil.	5	A.....	♂	(16)14	2–3	6.7	1	Sarcoma.....	11	6	Pulmonary tumor.	10.7
											1	Lymphocytic leukemia.	2
				♀	(14)12	2–3	11.2	0	.....	.....	7	Pulmonary tumor.	14.6
											1	Mammary tumor..	10

	.05 cc. olive oil	5	A	♂	16	2–3	11.7	0			4	Pulmonary tumor.	17
											2	Hepatoma	16
											1	Myoepithelioma	4
				♀	14	2–3	14.1	0			11	Pulmonary tumor.	15
											2	Lymphocytic leukemia.	12
											1	Myoepithelioma	10
VI	.05 cc. .05% soln. HS in olive oil.	6	C3H	♂	24	2–3	13.1	1	Rhabdomyosarcoma.	15	7	Hepatoma	19.6
VII	.025 mg. HN_2 in .25 cc. H_2O.	6	C3H	♂	(24)21	2–3	17	3	Sarcoma	15.3	8	Pulmonary tumor.	17.5
								2	Sarcoma—neurogenic in origin.	17	9	Hepatoma	16.4
											2	Hemangioendothelioma in the liver.	18.5
								1	Hemangioendothelioma.	20			
	Not injected		C3H	♂	(24)21	2–3	15.5				5	Pulmonary tumor.	15.2
											9	Hepatoma	17
VIII	.05 cc. .05% soln. HS in olive oil.	6	C3Hf	♂	(40)38	2–3	16.8	2	Sarcoma	14.5	5	Pulmonary tumor.	18.4
											22	Hepatoma	18.5
IX	.025 mg. HN_2 in .25 cc. H_2O.	6	C3Hf	♂	(41)37	2–3	16.1	2	Sarcoma	17	21	Pulmonary tumor.	17.8
								1	Papilloma	21	17	Hepatoma	18.8
											1	Reticulum-cell sarcoma.	23
											1	Lymphocytic leukemia.	21
											1	Hemangioendothelioma in liver.	17
	Not injected		C3Hf	♂	(40)39	2–3	16.3				6	Pulmonary tumor.	20
											18	Hepatoma	20.2
											2	Lymphocytic leukemia.	20.5
											1	Squamous-cell carcinoma of the skin.	19

*Average months after beginning of experiment.

sulfur mustard; one was in a strain A mouse injected with sulfur mustard; and 5 were in strains C3H and C3Hf mice injected with the nitrogen mustard. Two interesting subcutaneous sarcomas of neurogenic origin occurred in C3H males injected with the nitrogen mustard, and a subcutaneous rhabdomyosarcoma occurred in a C3H male injected with sulfur mustard. The three papillomas were probably of significance since they occurred at the site of injection of the nitrogen mustard, and the squamous-cell carcinoma that arose at the site of injection of the nitrogen mustard may also have been significant. This tumor had metastasized to the lung. The remaining two tumors occurring at the site of injection were a hemangioendothelioma in a C3H male injected with the nitrogen mustard and a mammary carcinoma in a C3Hf female injected with sulfur mustard.

Three of these induced tumors have been transplanted, since transplantation constitutes one of the tests for malignancy. The subcutaneous sarcoma that arose in the strain A male injected with sulfur mustard was transplanted through 13 transplant generations in strain A mice. It was a relatively slowly growing sarcoma requiring transplantation at intervals of approximately 42 days. The subcutaneous sarcoma that arose in the C3H male injected with sulfur mustard in experiment III has been transplanted in strain C3H through 44 transplant generations. This is a rather rapidly growing tumor requiring transplantation at intervals of from 14 to 21 days. One of the three subcutaneous sarcomas that arose in C3H males injected with nitrogen mustard in experiment VII has been transplanted in C3H mice through 25 transplant generations. This, also, is a rapidly growing tumor requiring transplantation at intervals of from 14 to 21 days. All of these tumors grew progressively to kill 100 percent of the animals receiving transplants. Thus, on the basis of successful transplantation, these three tumors that were tested would be considered malignant.

When the incidences of tumors occurring at sites other than the site of injection were considered, it was found that the incidence of pulmonary tumors was higher in the strain C3H and strain C3Hf mice injected subcutaneously with nitrogen mustard than in the controls. Experiment IX offered probably the best test in that the experimental and control mice were of comparable ages when autopsied. There was an incidence of pulmonary tumors of 56.8 percent in the injected animals as compared with an incidence of 15.4 percent in the controls. The difference between these two incidences is highly significant: $\chi^2=14.19$; $P<.001$. As in previous studies on pulmonary tumors in mice, the difference was also apparent when the average number of pulmonary tumors per animal was considered. The average number in the injected mice was .86 as compared with an average of .15 in the controls. The difference between these averages is also highly significant: $t=3.95$; $P<.01$.

Such a significant difference was also found when all the C3H and C3Hf mice injected with nitrogen mustard were compared with all their controls. It was probably permissible to combine the data from the C3H and C3Hf mice since strain C3Hf has not been found to deviate from strain C3H in

respect to incidence of pulmonary tumors. The incidence in the injected mice was 46.6 while that in the controls was 18.9. The difference between these incidences is highly significant: $\chi^2=12.78$; $P<.001$.

There was not, however, an increase in incidence of pulmonary tumors in the mice injected with sulfur mustard above that observed in the controls. In experiment V in which strain A mice were used, an incidence of 50 percent was observed in both the experimental and the control groups. In the C3Hf mice injected with sulfur mustard in experiment VIII there was an incidence of 13.2 percent with an average of .15 nodules per mouse, as compared with an incidence of 15.4 percent with an average of .15 nodules in the C3Hf controls listed under experiment IX. In all the C3H and C3Hf mice injected with sulfur mustard there was an incidence of pulmonary tumors of 8.4 percent as compared with an incidence of 15.8 percent in all the controls for these groups. None of the differences between these experimental and control groups is statistically significant.

For the most part the pulmonary tumors appeared histologically typical of those usually occurring in the mouse (*6*) and also of those in the guinea pig (*7*). An exception, however, occurred in a control C3H male in experiment VII which at 17 months after the beginning of the experiment was found to have 83 small pulmonary nodules. Histologically these appeared to be pulmonary tumors but were undergoing a sarcomatous change such as has been described as occasionally occurring in pulmonary tumors of the mouse, especially when transplanted (*8*). A similar change was also noted in one of the pulmonary tumors in a C3Hf male of experiment IX 20 months after injection with the nitrogen mustard. The only other marked variation noted was a deposit of calcium in one pulmonary tumor in a C3H male 23 months after injection with sulfur mustard, similar to such deposits noted in induced pulmonary tumors in guinea pigs (*7*).

Hepatomas were observed particularly in the strain C3H and strain C3Hf males, but in no experiment was there any evidence of an increase in the occurrence of this tumor due to either of the mustards. In all experiments the incidence observed in the experimental animals was the same or approximately the same as that in the controls.

Neoplasms of the blood-forming organs occurred in a number of the groups but did not occur in significantly greater numbers in those injected with either of the mustards than in the controls. One reticulum-cell sarcoma that occurred in a C3Hf male of experiment IX, 23 months after injection with the nitrogen mustard, was of particular interest histologically in that most of the neoplastic cells were multinucleate. Such cells are often seen in smaller proportions in this type of tumor.

A high incidence of mammary tumors was noted in the strain C3H females as was to be expected in females of this strain, but there was not a significant difference in the incidence found in those injected with the mustards compared with that in the controls. The occurrence of two mammary tumors in the 9 C3Hf females injected with sulfur mustard in

experiment IV may have been significant. Since these females were virgins, this incidence of 2 out of 9 may be contrasted with the occurrence of only 2 mammary tumors in a group of 100 C3Hf untreated virgin females of a concurrent study (*5*) that could serve as controls for this experiment.

A number of other tumors were observed and are listed in table 1 but none of these occurred with sufficient frequency for significance. The two myoepitheliomas observed were similar to those that have been described (*9*). The origin of these two appeared to have been in the salivary gland.

In addition to the neoplastic response, all the C3Hf mice injected with either the sulfur mustard or the nitrogen mustard showed graying of the hair over the injected area, such as reported by Boyland and Horning (*10*) in colored mice injected subcutaneously and intradermally with nitrogen mustards.

Discussion

In comparison with the induction of tumors at the site of injection of nitrogen mustard and sulfur mustard reported herein, Boyland and Horning (*10*) reported that in 14 mice injected subcutaneously with the nitrogen mustards methyl-bis and methyl-tris, 10 tumors arose and one of these arose at the site of the injection and was a sarcoma. In addition, Griffin *et al.* (*11*) reported fibrosarcomas, lymphosarcomas, and adenocarcinomas in mice and rats injected either subcutaneously, intraperitoneally, or intravenously with methyl-bis or methyl-tris, and, while the authors state that most of the tumors arose at sites other than the site of injection, it is assumed that at least some of the fibrosarcomas arose at the site of injection. No reference to any previous induction of tumors at the site of the subcutaneous injection of sulfur mustard has been found, although Berenblum (*12*) reported anticarcinogenic action of sulfur mustard when applied in conjunction with carcinogenic tar to the skin of stock mice.

While the number of tumors induced at the site of injection in the experiments reported herein was great enough to establish beyond doubt the carcinogenicity of the sulfur mustard and the nitrogen mustard when injected subcutaneously, the number was not as great as has often been reported following subcutaneous injection of the carcinogenic hydrocarbons. This, however, may be due to a difference in amount of the dose. Whereas a dose of 1 mg. of the hydrocarbons has been frequently used, since mice tolerate such a dose well, the total dose of either of the mustards used here did not exceed .2 mg. Furthermore, only a fraction of this dose could have been considered as the effective dose. The nitrogen mustard in water would have been expected to have been readily absorbed from the site of injection and to have been broken down within a short time.

While the sulfur mustard was in oil and therefore remained at the site of injection longer than the nitrogen mustard, in many of the animals injected with sulfur mustard, as well as those injected with the nitrogen mustard, ulcers appeared at the site of injection and, thus, some of the sulfur mustard was undoubtedly lost. Ulceration, however, did not preclude formation of tumors at the site of injection, for approximately half of these tumors arose in animals in which ulcers had appeared and had healed. In previous tests (*2, 3*) both compounds when injected intravenously gave a pulmonary-tumor response of a magnitude comparable with that from similar amounts of carcinogenic hydrocarbons injected intravenously.

Whereas both the sulfur mustard and the nitrogen mustard induced pulmonary tumors when injected intravenously, when injected subcutaneously in the present experiments only the nitrogen mustard increased the occurrence of pulmonary tumors. This may have been due to the fact that the nitrogen mustard, being in water, was more readily carried to the lungs than was the sulfur mustard, which was in oil. Furthermore, as indicated above, some of the sulfur mustard may have been lost. However, it has been noted that the hydrocarbons are much more effective in inducing pulmonary tumors when injected intravenously than when injected subcutaneously. It should be emphasized that the induction of pulmonary tumors with the nitrogen mustard in these experiments was in strain C3H and strain C3Hf mice, which were genetically more resistant to pulmonary tumors than were the strain A mice in which the increase in occurrence of pulmonary tumors was previously noted following intravenous injection of these mustards.

Summary

Sulfur mustard, bis(2-chloroethyl) sulfide, when injected subcutaneously into strains C3H, C3Hf, and A mice, induced neoplasms at the site of injection as did also the nitrogen mustard, methyl bis(2-chloroethyl) amine hydrochloride, injected subcutaneously into strains C3H and C3Hf mice. The tumors occurring at the site of injection included 11 sarcomas that were probably fibrosarcomas, 2 sarcomas neurogenic in origin, a rhabdomyosarcoma, 3 papillomas, a squamous-cell carcinoma, a hemangioendothelioma, and a mammary carcinoma.

Three of the sarcomas were transplanted and have been successfully carried in the strain of origin through many transplant generations.

Neoplasms occurring at sites other than the site of injection are listed, as are also the neoplasms occurring in the controls. There was a significant increase in the occurrence of pulmonary tumors in the strains C3H and C3Hf mice injected subcutaneously with the nitrogen mustard over that in the controls.

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An Instrument for Gastric Biopsy of the Rhesus Monkey [1]

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Certain studies of experimental carcinogenesis indicate that fat-soluble and water-insoluble carcinogenic hydrocarbons can be brought into clear and homogenous *aqueous* solution with the aid of certain lipophilic and hydrophilic substances (*1–3*), and that these substances enhance the absorption of carcinogenic hydrocarbons into the wall of the normal glandular mucosa of the stomach (*4–8*). These experiments also showed that the solubilization mechanism of these substances has properties in common with factors participating in normal intestinal absorption of fats, such as fatty acid combined with bile acid salts, some bile acids, cholesterin, etc. (*2, 3, 6–8*). A characteristic of these colloids is that when dissolved in water their molecules and ions aggregate (when a certain concentration is exceeded) to form micelles that are able to solubilize considerable amounts of fats and fat-soluble substances. We are now continuing our studies with the working hypothesis that gastric adenocarcinoma may occur in the stomach in which there is a "functional and anatomic intestinalization" (*6, 8*); in other words, when factors participating in fat absorption are present, and a suitable tissue of origin for malignant transformation exists, adenocarcinoma may develop.

In studies of experimental gastric carcinogenesis it is important to follow continuously the histogenetic changes taking place in the gastric glandular mucosa in *one and the same animal* without injuring or killing it. We employed equipment, constructed by ourselves, by means of which the gastric mucosa of living rhesus monkeys can be examined in a simple manner. The biopsy forceps utilized for gastrobiopsy of human beings (*9*) cannot be used on monkeys because of the thinness of the stomach wall. The caliber of the esophagus of the monkey is small. Other types of biopsy instruments based on the suction principle (*10, 12–15*) have been described recently, and these can also be used to obtain biopsy specimens of experimental animals by remodeling them in an appropriate manner. The advantage of a suction biopsy of this kind lies in the fact that it is rather safe. For that reason we have constructed

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for experimental purposes a device that is partially based on the suction principle. In addition, we have devised a similar instrument for human biopsy.

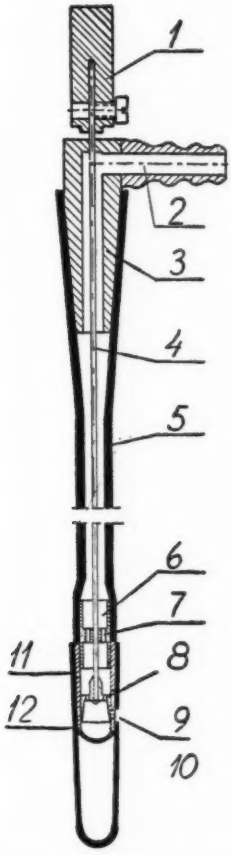
The instrument we employed for the rhesus monkeys (text-fig. 1) was made from a rubber tube, measuring about 40 cm. in length. The distal end of this device was equipped with a metal, olive-shaped structure with a rounded and detachable head, on the side of which a round hole (measuring from 1.4 to 2.0 mm. in diameter) is provided. A cylindrical knife is mounted within the "olive," and this is operated by a steel coil running inside the rubber tube. The apparatus is connected with a vacuum drain, the vacuum pressure of which may be adjusted as occasion may require. When the instrument is introduced into the stomach, the suction is connected at the same time that the round hole is opened by pulling the cylindrical knife backward. At this point, a knuckle of tissue comprising a portion of the gastric mucosa and submucosa is sucked up via the hole into the "olive." By pushing the knife forward, the desired specimen can be cut off from this knuckle. Rotation of the instrument brings the various portions of the gastric wall into the hole. Although no anesthetic whatever is necessary, it is beneficial to use a drug such as isoamylpropionate given intravenously. It is not necessary to perform lavage of the gastric cavity or to evacuate the gastric secretion.

In the present experiments the monkeys were allowed to fast only 8 to 12 hours prior to the biopsy procedure. Immediately after the biopsy, the animals were given food *ad libitum*. No complications resulted from this procedure, notwithstanding the fact that about 200 biopsies from 30 different monkeys have been taken over a period of one year. Satisfactory tissue specimens can likewise be obtained from the gastric mucosa when there are signs indicating the presence of different types of gastritic lesions. The only disadvantage is, perhaps, that an exact localization is not possible. However, by palpating the abdominal wall, it is possible to distinguish the location of the metal "olive" as being in the antrum, corpus, or fundus of the stomach. Furthermore, the ventral and dorsal walls may be easily distinguished. Five specimens have been obtained from the gastric mucosa of one monkey in one experiment.[3]

The instrument we made for use on human beings is in principle similar to the one described for monkeys. It is, however, only semiflexible, while the device constructed for animals is fully elastic. The lengths of the flexible and rigid parts of our apparatus correspond to those of the gastroscope described by Schindler (*16*). This device differs, for instance, from that of Tomenius (*12, 13*) in that its diameter is considerably smaller and the flexibility of its elastic part is due to a steel tube 1.8 mm. in diameter that runs through the rubber tube, within which is a thin steel coil to operate the cylindrical knife. About 500 biopsy specimens have been taken from human beings (*11*).

[3] Since the present paper was written, gastric alterations have developed in the stomach of some monkeys. A full account of these and related observations will be given later.

Histologically, the biopsy specimens proved to be successful with the devices constructed for both animals and human beings (*cf.* figs. 1 and 2).



TEXT-FIGURE 1.—Instrument for gastrobiopsy of the rhesus monkey.

Summary

A simple suction-type biopsy instrument is described for obtaining biopsy specimens of the gastric mucosa of rhesus monkeys, no matter how frequently, without injury to the animal. By this means it is possible to follow changes in the gastric mucosa during experimental treatments involving one and the same animal. In addition, a similar instrument has been constructed for human beings. The biopsy specimens are histologically excellent. Devices based on this suction principle can be made to obtain biopsy specimens of the gastric mucosa from smaller animals as well.

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PLATE 11

FIGURE 1.—Biopsy specimen from the wall of the stomach of a normal monkey. Hematoxylin and eosin. × 180

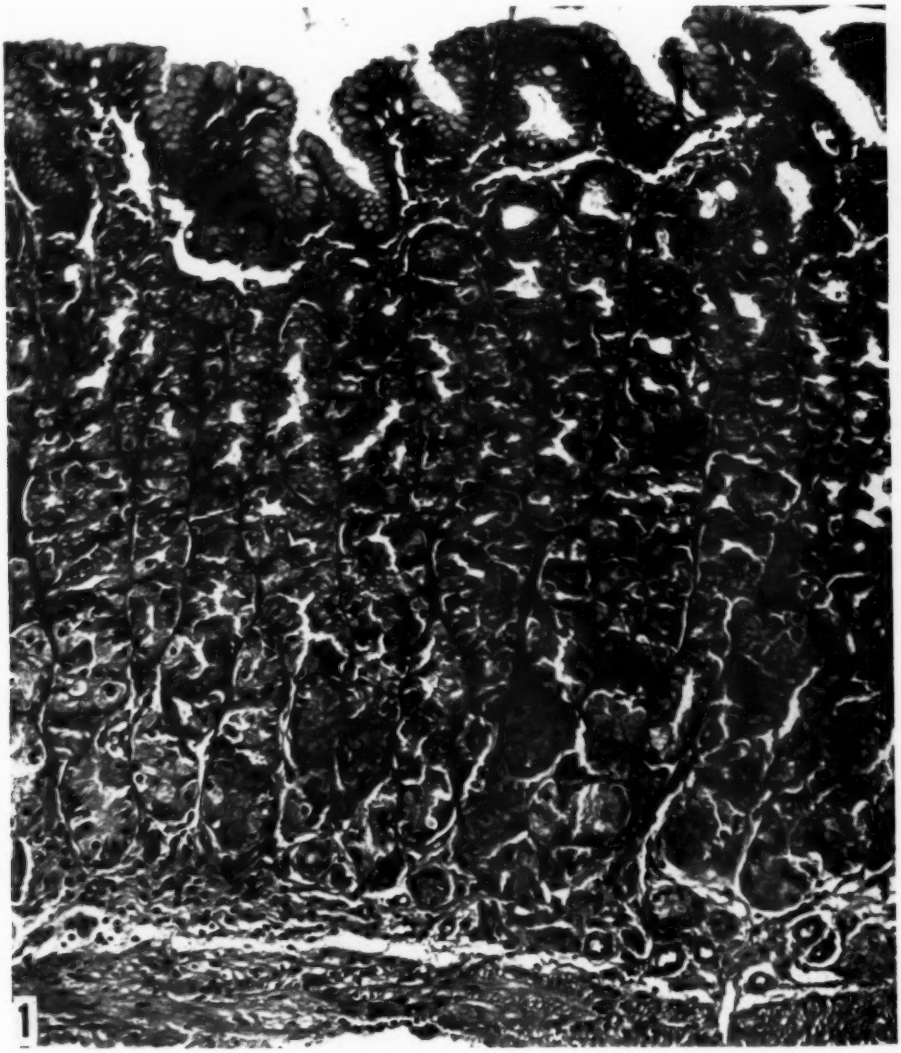

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PLATE 12

FIGURE 2.—Biopsy specimen from the wall of a human stomach. So-called atrophic gastritis with intestinal metaplasia, goblet cells, etc. Hematoxylin and eosin. × 180

2

Neoplasms in Rats Exposed to Single-Dose Total-Body X Radiation [1, 2]

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Malignant neoplasms have been induced in experimental animals by X radiation since 1910, when Clunet caused a sarcoma in a rat by this means. Since then most investigators have used repeated X-ray exposure of cancer-susceptible mice as the experimental method (*1*). Furth and Furth (*2*) increased the incidence of ovarian tumors, lymphomatosis, and myelosis to a significant degree by irradiation of mice using single or repeated doses. The appearance of a fibrosarcoma in a mouse of non-cancerous strain, 10 months after exposure to a single dose of X radiation, has been described (*3*).

The present observations on rats that have survived lethal amounts of X radiation are made possible by the protection from lethal radiation effects afforded by postirradiation parabiosis (*4–6*). Temporary surgical union (for 30 days or less) with a littermate partner following irradiation provides an interchange of blood that maintains the irradiated animal during the critical 5 to 10 days after exposure (*7*).

Procedure and Results

Female Holtzman littermate rats weighing from 115 to 125 gm. were used in this experiment. The rats to be irradiated were placed in small cardboard boxes 20 × 6 × 5 cm. in size. Two boxes, each containing one rat, were centered in the 20 × 20 cm. field. In every case, total-body irradiation was directed to the dorsal aspect of the animal. Irradiation was delivered by a GE-Maxitron with the following factors: 250 kv., 30 ma., 0.25 Cu and 1.0 Al added filter, H.V.L. 0.88 Cu, 50 cm. F.S.D., 20 × 20 cm. field, intensity 127 r per minute. The rats were irradiated with 700 r (in air), the $LD_{95}/30$ days in this laboratory, and within three hours were placed in parabiosis with nonirradiated female litter mates. Eighty percent of the animals survived for at least 30 days, when they

[1] Received for publication March 27, 1953.
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[3] With the technical assistance of James K. Butler.

were separated surgically and kept for observation of any late effects of the single exposure to this high dose of ionizing radiation.

Thirty-two pairs have been observed for more than 6 months and no malignant growths have been found in the nonirradiated partners. However, 9 of the 32 irradiated partners have developed rapidly growing tumors. The distribution of these tumors by location is illustrated in text-figure 1, and a description of each follows:

1) *Irradiation and parabiosis 8/18/52; surgical separation 9/18/52.*—The irradiated partner died 1/2/53, as a result of a 40-gm. tumor attached to the scapula (text-fig. 1-A)—an invasive mass of straplike cells with many normal and abnormal mitotic figures and typical cross-striations in the cytoplasm—diagnosed a rhabdomyosarcoma (figs. 1, 2). On the left flank of the same rat a moderate-sized tumor was found (text-fig. 1-A), with columns and cords of columnar cells largely in acinar formation, actively growing and with an appearance suggestive of invasion through its capsule. It was known to have grown rapidly and is considered an adenocarcinoma (fig. 4). Transplants of the rhabdomyosarcoma grew in recipient rats and the tumor is being maintained.

2) *Irradiation and parabiosis 8/18/52; surgical separation 9/18/52.*—The irradiated partner appeared emaciated and moribund 1/14/53; at autopsy a tumor mass was found in the cranial cavity (text-fig. 1-B), composed largely of whorls of elongated spindle-shaped cells with a slight tendency to palisade, invading the calvarium and having in one area of the section cells of smaller size, chronic inflammatory-cell infiltration, and the presence of some acinar structures that may be nasal sinus epithelium which has become surrounded and isolated. It is probably a meningioma (fig. 3).

3) *Irradiation and parabiosis 9/24/52; surgical separation 10/3/52.*—Biopsy 1/15/53 from the left groin of the irradiated partner (text-fig. 1-C) showed a glandular tumor with an appearance suggestive of invasive tendency—an adenocarcinoma.

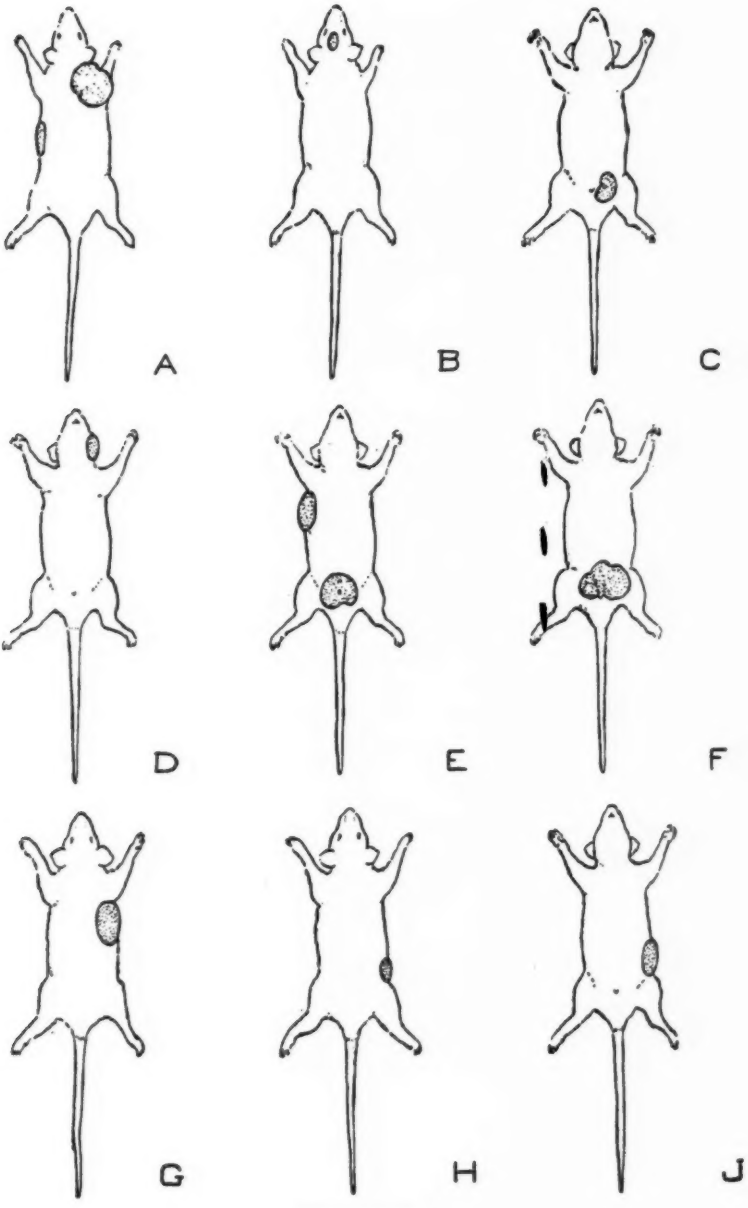
4) *Irradiation and parabiosis 8/18/52; surgical separation 9/18/52; biopsy 1/23/53; autopsy 2/6/53.*—A tumor mass, diagnosed by biopsy as a typical basal-cell carcinoma (fig. 5), was growing rapidly on the left cheek of the irradiated partner (text-fig. 1-D). No metastases were found at autopsy.

5) *Irradiation and parabiosis 8/18/52; surgical separation 9/18/52.*—Biopsy, 1/23/53, revealed a large, rapidly growing well-differentiated adenocarcinoma along the midline of the ventral body wall, involving the clitoris and occluding the vaginal opening (text-fig. 1-E).

Biopsy, 3/12/53, showed a tumor of the right flank (text-fig. 1-E) consisting of well-formed glandular acini surrounded by many layers of fibrous tissue—a pericanalicular fibroadenoma.

TEXT-FIGURE 1.—Schematic diagram to illustrate the location of tumors that developed within 6 months after 6-week-old female rats had been exposed to 700 r, and protected from death by parabiosis.

A. (dorsal view)—rhabdomyosarcoma of scapular area, adenocarcinoma of the left flank.
B. (dorsal view)—meningioma.
C. (ventral view)—adenocarcinoma.
D. (ventral view)—basal-cell carcinoma.
E. (ventral view)—midline adenocarcinoma, pericanalicular fibroadenoma of right flank.
F. (ventral view)—adenocarcinoma.
G. (dorsal view)—fibrosarcoma.
H. (dorsal view)—adenoma.
J. (ventral view)—pericanalicular fibroadenoma.



TEXT-FIGURE 1.

6) *Irradiation and parabiosis 8/18/52; surgical separation 9/18/52.*—Biopsy, 1/23/53, disclosed a well-differentiated adenocarcinoma of the midventral abdominal wall (text-fig. 1-F) known to have grown rapidly, with frequent mitotic figures and an appearance suggestive of invasion of the overlying tissue.

7) *Irradiation and parabiosis 8/18/52; surgical separation 9/18/52.*—Biopsy, 3/12/53, revealed a tumor on the right flank (text-fig. 1-G) consisting of a mass of polyhedral and spindle-shaped cells with oval nuclei and frequent mitosis. The tumor is highly vascular and, in a few places, tumor cells can be seen in vascular spaces—a fibrosarcoma (fig. 6).

8) *Irradiation and parabiosis 8/18/52; surgical separation 9/18/52.*—Biopsy, 3/12/53, revealed a tumor having the appearance of a chronically inflamed, somewhat active adenoma, probably arising from sebaceous-gland elements (fig. 7). This tumor of the right hip (text-fig. 1-H) grew to a size of 1.5 × 1.0 cm. in a week, so that the possibility of malignancy must be considered.

9) *Irradiation and parabiosis 8/18/52; surgical separation 9/18/52.*—Biopsy, 3/12/53, of a large tumor of the left ventrolateral flank (text-fig. 1-J) revealed well-formed glandular acini surrounded by many layers of fibrous tissue—a pericanalicular fibroadenoma (fig. 8).

All these tumors have appeared within 6 months after irradiation in rats less than 8 months of age. The surviving rats will be observed during their subsequent life span for tumor incidence and other pathologic manifestations.

Discussion

Spontaneous malignant tumors are rare in most of the ordinary strains of laboratory rats, especially during the first year of life (*8*, *9*). Ratcliffe (*8*) found an incidence of 3.1 percent of deaths from tumors over a five-year period in two colonies of Wistar Institute rats (6.7% of females; 1.2% of males). The age of onset of these tumors was 120 to 1,020 days and fewer than 4 percent of the tumors developed in rats less than 9 months of age. Curtis, Bullock, and Dunning (*9*) found 1.3 percent deaths from tumors in 31,868 rats of seven distinct strains. The age of death of tumor-bearing female rats in this series was from 5 to 36 months, with the greatest tumor incidence occurring at 21 months.

The tumors observed in the rats in the present study are believed to be related to the previous irradiation.[4] They appeared in a high percentage of our rats, at an age when tumors are not usually present, and were found only in the irradiated animals.

Tumors have recently been found in rats that were maintained in parabiosis for 9 months or more (*10*). However, our rats were in parabiosis for only 30 days or less and tumors developed only in the irradiated partners, 4 to 6 months after surgical separation. None of the neoplasms in our irradiated rats has been lymphoid in origin.[5]

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[4] Since our paper was submitted, Koletsky and Gustafson (Proc. Am. Assoc. Path. & Bact., April, 1953) have reported similar tumor induction within 15 months in those rats that survived an LD_{70}/30-day dose of total-body radiation.

[5] The absence of lymphomas in these parabionts may possibly be related to the observations that spleen protection, bone-marrow injections, and thigh-shielding of irradiated mice also inhibit subsequent development of lymphoid tumors (KAPLAN, H. S., and BROWN, M. B., J. Nat. Cancer Inst. 12: 427–536, 1951; KAPLAN, H. S., BROWN, M. B., and PAULL, J., Proc. Am. Assoc. Cancer Research 1: 28, 1953; LORENZ, E., CONGDON, C. C., and UPHOFF, D., Proc. Am. Assoc. Cancer Research 1: 34, 1953).

The principal contribution of a study such as this is to determine the late effects of what would normally be a lethal dose of X radiation in animals protected from death by postirradiation parabiosis.

Summary

Eleven tumors of various types have been observed in 9 female rats of 32 that had been irradiated with a lethal dose of X radiation and protected from death by postirradiation parabiosis. The neoplasms developed within 6 months after irradiation in rats less than 8 months of age, and none has appeared in the nonirradiated partners. Observation for further tumor incidence is to be continued throughout the life span of the animals.

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PLATE 13

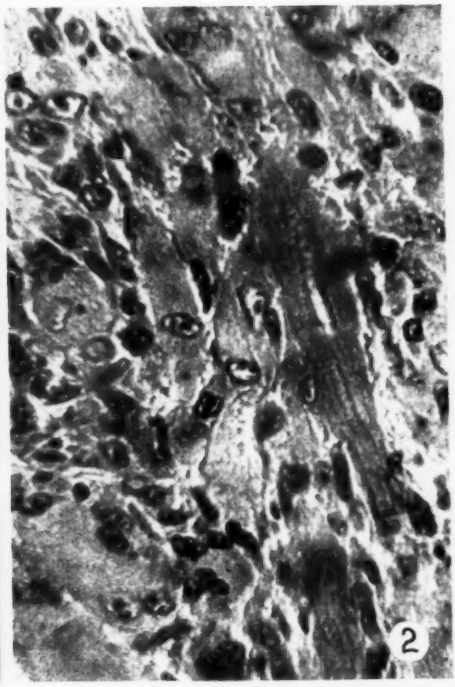
FIGURE 1.—Microscopic section of the rhabdomyosarcoma, attached to the scapular region in rat #1. × 100

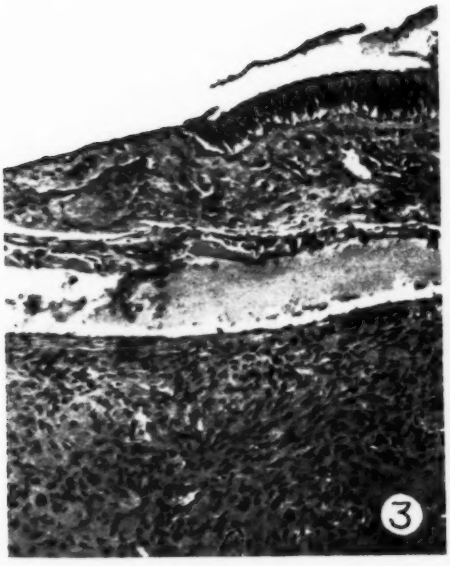
FIGURE 2.—Same as figure 1. × 430

FIGURE 3.—Microscopic section of the meningioma invading the nasal sinuses found in rat #2. × 100

FIGURE 4.—Microscopic section of the adenocarcinoma from the left flank of rat #1. Similar tumors were also found in rats #3, #5, and #6. × 100

All preparations were stained with hematoxylin and eosin.





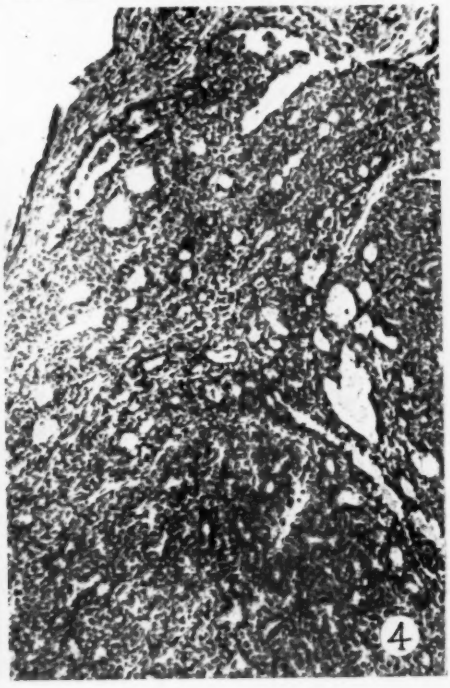


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PLATE 14

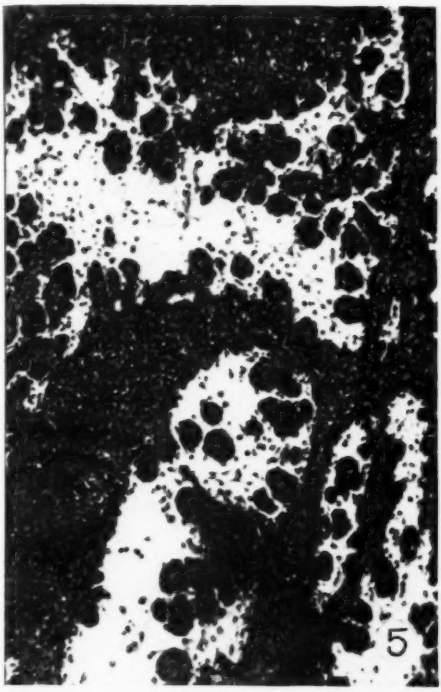
FIGURE 5.—Microscopic section of the basal-cell carcinoma that developed on the left cheek of rat #4. × 100

FIGURE 6.—Microscopic section of the fibrosarcoma of the right flank from rat #7. × 800

FIGURE 7.—Microscopic section of the adenoma on the right hip of rat #8. × 100

FIGURE 8.—Microscopic section of the pericanalicular fibroadenoma of the left ventrolateral flank of rat #9. A similar tumor formed on the right flank of rat #5. × 100

All preparations were stained with hematoxylin and eosin.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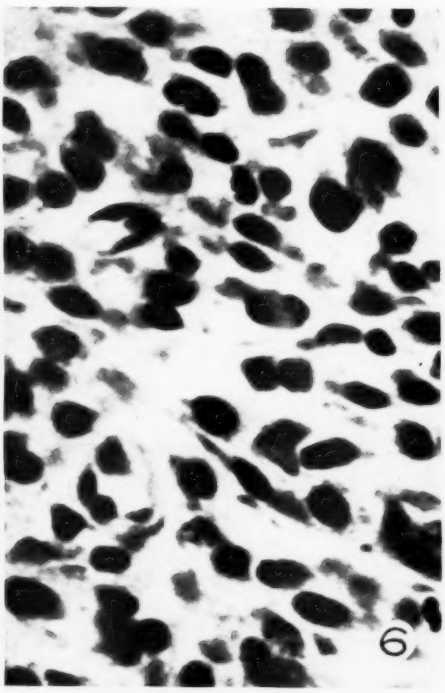
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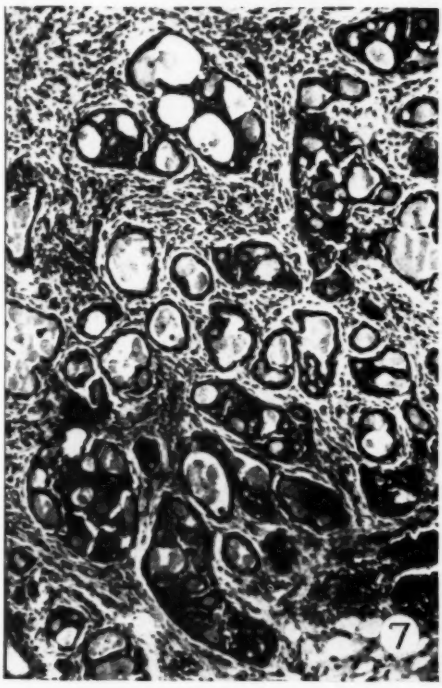
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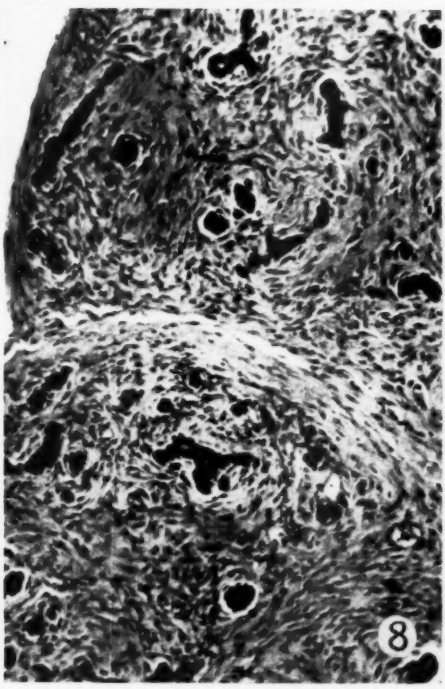
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Pages 159 to 175 (August 1953 issue): In the paper entitled "Neoplasms in Rats Protected Against Lethal Doses of Irradiation by Parabiosis or *Para*-Aminopropriophenone," by Brecher, Cronkite, & Peers, *for* "*para*-aminopropriophenone" *read* "*para*-aminopropiophenone."

Neoplasms in Rats Protected Against Lethal Doses of Irradiation by Parabiosis or *Para*-Aminopropriophenone[1]

G. Brecher, M. D.,[2] E. P. Cronkite, M. D.,[3,4] *and* James H. Peers, M. D.,[3] *Laboratory of Pathology and Pharmacology, National Institute of Arthritis and Metabolic Diseases,[2] and the Naval Medical Research Institute,[3] Bethesda, Md.*

This paper describes a number of neoplasms observed in rats that were treated with *para*-aminopropriophenone prior to exposure to lethal doses of irradiation and in rats that were joined in parabiosis before or after such exposure. The number of animals involved is too small to permit any conclusions as to the incidence of these tumors. Their histologic characteristics are believed worth reporting, however, because spontaneous occurrence of the particular tumors observed is extremely rare. Moreover, it is hoped that this report may stimulate long-term observation of laboratory animals protected from the immediate effects of lethal doses of radiation.

Materials and Methods

Groups of rats were exposed to single whole-body irradiation with doses ranging from 700 to 1,000 r. A number of these animals were protected against the early lethal effects of these doses by administering *para*-aminopropriophenone or glutathione before irradiation, or by joining them to nonirradiated litter mates before or after exposure to X ray. The survivors were observed until death or development of symptoms of disease. Details of the experiments are described below.

All animals were female rats of the Sprague-Dawley strain raised at the National Institutes of Health. Radiation was delivered by a Picker therapy X-ray machine operating at 200 KVP (25 ma. 1.0 mm. Al, 0.25 mm. Cu, TSD 50 cm., field size 20 × 20 cm., HVL 0.8 mm. Cu, dose rate 44 r in air per minute). Half of each total dose was delivered to the dorsal, and half to the ventral surface of the rat. Under these conditions the dose delivered to any part of a rat probably does not vary more than 1 percent. Actual measurements at various points of a masonite dummy differed by

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] National Naval Medical Center, Department of the Navy.

[4] The opinions and assertions are those of the authors and are not to be construed as reflecting the policy or opinions of the Naval Service.

only ±0.4 percent. All rats were 40 to 60 days old when irradiated, except those of the fifth experiment which were four months old.

In the first experiment, 20 rats weighing 115 to 148 grams were given an intraperitoneal injection of a 1 percent solution of *para*-aminopropriophenone in propylene glycol. The individual dose was 30 mg. per kilogram of body weight (*1*). Twenty minutes later, these rats were exposed to 700 r whole-body irradiation. Of the 20 animals injected with *para*-aminopropriophenone, 11 survived beyond the 30th postirradiation day. Of these 11 survivors, 6 died between the 30th and 60th postirradiation day. The remaining 5 animals lived more than six months. Twenty-eight rats of comparable age and weight were exposed to the same dose of irradiation without prior administration of *para*-aminopropriophenone. None survived beyond the 12th postirradiation day.

In the second experiment, 18 rats weighing 115 to 140 grams received 3 mg. of glutathione per gram of body weight intraperitoneally 15 minutes prior to irradiation with 700 r (*2*). Of the 18 animals treated with glutathione, eight survived beyond the 30th postirradiation day; one died at 45, one at 68, one at 93, and one at 150 days following exposure. The remaining four lived more than six months. Eighteen rats were given the same dose of irradiation without prior administration of glutathione. None of these survived beyond the 10th postirradiation day.

In a third experiment, 11 rats weighing 85 to 117 grams were exposed to 700 r and then joined in parabiosis to nonirradiated litter mates (*3*). Six of the parabiotic pairs survived 30 days and were separated at that time. One of these irradiated animals died 45 days and one 111 days after exposure; the remaining four lived more than six months following irradiation. Ten rats of comparable age and weight were irradiated at the same time but not joined in parabiosis, and none of them survived beyond the 15th postirradiation day.

In a fourth experiment, 12 rats weighing 150 to 190 grams were exposed to 800 r and then joined in parabiosis to nonirradiated litter mates. Eight pairs survived for 30 days and were separated at that time. One irradiated animal died on the 34th postirradiation day. The remaining 7 exposed rats lived more than six months. Ten rats of comparable weight and age were similarly irradiated but not joined in parabiosis, and none survived beyond the 12th postirradiation day.

A fifth experiment comprised 8 pairs of litter mates joined in parabiosis one month before irradiation. One partner of each pair was exposed to 1,000 r. Five of these pairs were killed for histologic studies within the first 5 days following exposure. The three remaining pairs were separated 16 days after exposure. One of the irradiated animals became progressively more anemic and was killed on the 20th postirradiation day. The two remaining pairs lived more than six months.

Results

The tumors found in rats that survived doses of 700 to 1,000 r for more than six months are summarized in table 1. No tumors were seen in any

TABLE 1.—*Tumors in animals surviving more than 6 months after whole-body irradiation with 700 to 1,000 r*

Dose in r	Treatment	No. of animals treated	No. of animals surviving 6 months or more	No. of animals with tumors	Type of tumors	Time between irradiation and autopsy
700	pre-irradiation, *p*-aminopropriophenone....	20	5	3	1 adamantinoma..... 1 renal carcinoma..... 1 subcutaneous sarcoma.....	6 months 10 months 11 months
700	pre-irradiation, glutathione.....	18	4	0		
700	postirradiation, parabiosis.....	11	4	1	adenocarcinoma of jejunum.....	16 months
800	postirradiation, parabiosis.....	12	7	3	1 glioma..... 1 adenocarcinoma of ileum..... 1 adenocarcinoma of duodenum.....	15 months 11 months 11 months
1000	pre-irradiation, parabiosis.....	*8	2	1	adenocarcinoma of colon.....	12 months
0	parabiosis.....	10	10	1	sarcoma of uterus.....	(16 months)†

*5 pairs were killed for histologic study within the first 5 days following irradiation.
†Time between irradiation of litter mates and autopsy.

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of the rats that died earlier. Fibroadenomas of the breast, which were common in both irradiated animals and untreated litter mates, are not included in table 1 or in the subsequent discussion. The increased incidence of these tumors following whole-body irradiation has recently been described by Passonneau *et al.* (*4*).

Only two of the tumors were identified by inspection or palpation during life. Four animals exhibiting weight loss, diarrhea, or bleeding from the vagina were killed with the expectation of obtaining tumor tissue suitable for transplantation, but no tumors were found. The remaining irradiated animals died spontaneously or were killed when moribund. In four of the irradiated animals, death was caused by extensive tumor growth in vital organs. The immediate cause of death of the remaining animals was bronchopneumonia. The frequency with which irradiated animals developed pneumonia was in contrast to the rarity of such infections in animals of comparable age kept for other experimental purposes in adjacent cages. This apparent increase in susceptibility to infection was not due to a depression of the bone marrow, as was determined from study of the bone marrow, the peripheral blood count, and the presence of massive leukocytic infiltration in the lung tissue.

Gross and Microscopic Findings in 8 Tumor-Bearing Irradiated Rats

Adenocarcinoma of jejunum.—Path. No. 57379. Whole-body irradiation, 700 r, December 27, 1950. Postirradiation parabiosis for 30 days. This rat developed a large mammary fibroadenoma and was killed on April 29, 1952, 490 days after irradiation, when the tumor had grown to a size of $10 \times 8 \times 6$ cm. Eight inches below the pylorus, the jejunum was markedly dilated, measuring 2.5 cm. in diameter. At the caudal end of the dilated portion of the gut, the normal mucosa abruptly changed to a partly thickened area consisting of irregularly proliferating glands and villi lined by hyperchromatic cells having numerous mitoses extending to the tips of the villi and without the usual regular differentiation of goblet cells (fig. 1). Mucus production was, however, evident in some of the dilated glands that had invaded the mesentery. An adjacent mesenteric extension of the tumor consisted of a large cyst lined by columnar epithelium having multiple papillary ingrowth (fig. 2). There were no metastases to the regional nodes or other organs.

Adenocarcinoma of the ileum.—Path. No. 57363. Whole-body irradiation, 800 r, May 4, 1951. Postirradiation parabiosis for 30 days. Killed March 28, 1952, 330 days after irradiation, because of marked enlargement of the abdomen. Beginning at about 8 cm. proximal to the ileocecal valve, the ileum was markedly dilated for a distance of approximately 5 cm. and contained a polypoid tumor. The tumor infiltrated through the wall and extended in the form of a narrow cord to the bifurcation of the uterus, and along the uterine horns to both ovaries, which were largely replaced by tumor tissue. The tumor also invaded the pancreas and the adjacent splenic capsule. The polypoid tumor of the ileum consisted of glandlike cystic spaces of varying size, lined by columnar epithelium and partly filled with albuminous material with an admixture of neutrophilic leukocytes near the ulcerated surface. The tumor was sharply demarcated from the adjacent intact mucosa by the abnormal configuration of the glandular spaces, but their epithelial lining closely resembled the normal intestinal epithelium, containing both goblet and Paneth cells (fig. 3). An inflammatory reaction was seen only at or near the surface of the primary tumor, which had penetrated and replaced the entire muscularis, producing a marked fibroblastic reaction with osteoid formation. The secondary tumor deposits closely resembled the primary lesion except for the generally smaller size of the glandlike structures and occasional

proliferation of solid cords or nests of tumor cells (fig. 4). Even in these areas, however, and in groups of tumor cells invading lymphatics or veins, their differentiation into goblet or Paneth cells was frequently maintained.

Adenocarcinoma of the duodenum.—Path. No. 57371. Whole-body irradiation, 800 r, on May 5, 1951. Postirradiation parabiosis for one month. Died April 17, 1952, 350 days after irradiation. At autopsy, a tumor mass was found in the abdomen that replaced the mucosa of both the pyloric portion of the stomach and the first part of the duodenum and extended through the diaphragm, compressing and invading both lungs. Tumor deposits were also present along the anterior aspect of the thoracic vertebral column and in the pancreas. The structure of this tumor was indistinguishable from the one in the ileum described above. The primary site could not be determined anatomically either at autopsy or after serial sectioning of the intestinal tumor. However, the differentiation of invasive tumor tissue into Paneth cells suggested the duodenum rather than the stomach as the site of origin.[5]

Adenocarcinoma of the colon.—Path. No. 60526. Whole-body irradiation, 1,000 r, January 8, 1952. Pre-irradiation parabiosis. Separation 14 days after irradiation. Killed in moribund state, December 23, 1952, 350 days after irradiation. At autopsy bilateral bronchopneumonia was present. In the colon there was a small sessile polyp, about 5 mm. in diameter, consisting of irregular glands lined with hyperchromatic columnar epithelium without any differentiation into goblet cells (fig. 5). The muscularis mucosae was interrupted at the narrow base of the polyp and partly reflected at its edge. Irregular glandular structures extended down to, but did not invade, the muscular wall.

Glioma.—Path. No. 59169. Whole-body irradiation, 800 r, on May 5, 1951. Postirradiation parabiosis for one month. This rat developed intermittent convulsive movements and was killed on August 11, 1952. There was an extensive hemorrhage in the left frontal lobe, secondary to a tumor that was recognized only upon microscopic examination (fig. 6). The main mass of the tumor consisted of closely packed cells with ample cytoplasm of ill-defined outline and nuclei of fairly uniform size, varying from round or oval to kidney-bean and irregular shapes, with numerous mitotic figures. Beyond the main mass, tumor cells permeated the adjacent brain substance in which ganglion cells were still intact (fig. 7), and extended along perivascular spaces and beneath the meninges.

Renal carcinoma.—Path. No. 59170. Whole-body irradiation, 700 r, October 12, 1951, following administration of *para*-aminopropriophenone. An abdominal tumor was palpable, and the animal was killed on August 25, 1952, 320 days after irradiation. The palpable tumor was located in the perirenal tissue and there were numerous tumor nodules in both kidneys, both lungs, and the mesentery. Solitary tumor nodules were present in the liver and the serosa of the jejunum. Both adrenal glands were invaded by tumor tissue. The masses in the kidney included circumscribed multicystic lesions that contained albuminous fluid with varying numbers of red blood cells. The cysts were lined partly with a single or double layer of cuboidal epithelium and partly with stratified transitional epithelium devoid of intercellular bridges (fig. 8). Other cystic lesions showed multiple papillary projections lined with stratified epithelium of the transitional type, with superficial keratinization in one area. Elsewhere the kidney contained solid nests of round or oval cells resembling the cuboidal cell layer of some of the cystic lesions; and there was invasion of glomeruli or individual glomerular loops by similar tumor cells (fig. 9). The renal pelvis was not invaded in any of the numerous sections examined and there was no evidence of pyelitis. The pulmonary nodules of tumor tissue consisted of cystic lesions with papillary projections of connective-tissue stalks covered by stratified epithelium, and of similar papillary growth without cyst formation. The stratified epithelium of the tumor frequently showed extensive superficial keratinization. Portions of the epithelium of smaller bronchi were frequently replaced by stratified epithelium (fig. 10). Such partial re-

[5] In a recent, still incomplete series of experiments, another intestinal adenocarcinoma of the same type, with extensive lymphatic spread, was found in a parabiotic rat 6 months after exposure to 900 r.

placement of the bronchial epithelium was always associated with adjacent extensive tumor growth, which often extended beneath intact bronchial epithelium. The metastases in the liver, the mesentery, and in the cervical lymph nodes (fig. 11) showed cystic and papillary tumor growth similar to that seen in the kidney. Keratinization was not seen in any of these metastatic lesions.

The primary site of the tumor could not be established. The possible diagnosis of primary tumor of the lung was considered, but the multicystic lesions of the kidney with their cuboidal epithelium were quite unlike the structure of metastases of known pulmonary tumors. A diagnosis of renal carcinoma was therefore made. The superficial keratinization in the pulmonary lesions was considered to be squamous metaplasia. This tumor was successfully transplanted subcutaneously in rats for three generations and maintained its cystic papillary growth pattern. No squamous metaplasia was present in any of the transplants. One of the subcutaneous transplants grew to 3 cm. in diameter over a period of two months, but failed to metastasize.

Adamantinoma.—Path. No. 57380. Whole-body irradiation, 700 r, October 12, 1951, following administration of *para*-aminopropriophenone. About 6 months later, a slowly enlarging tumor of the left side of the lower jaw was noted and the animal was killed on April 29, 1952, 200 days after irradiation. The left side of the mandible was partly replaced by a cyst measuring 2 cm. in diameter, with a partly calcareous wall, lined with soft tissue, measuring up to 5 mm. in thickness. On section, the tumor consisted of cords and nests of epithelial cells with palisading of the marginal cells, the nuclei of which often stood away from the adjacent connective tissue stroma (fig. 12). Eccentrically situated in nests of tumor cells were spicules of homogeneous oxyphilic material that occasionally showed fine striations resembling those of dentin. More frequently, this material was perforated by capillaries and resembled dental cement. With Heidenhain's azan stain, this material was colored blue. Contiguous tumor cells often formed a layer of very tall columnar cells containing oxyphilic granules which stain brilliant red. A fine line of similarly staining material was often present on the adjacent cement- or dentin-like material (fig. 13). Rywkind (*5*) has described a similar tumor in man and has interpreted the secretory activity of the tumor cells as representing an unsuccessful attempt at enamel formation, which normally does not result in accumulation of secretory granules in the ameloblasts. Although the tumor formed dentin and cement, it has been classified as adamantinoma since the bulk of the tumor was epithelial and duplicated in structure the usual adamantinoma seen in man.

Sarcoma of subcutis.—Path. No. 59415. Whole-body irradiation, 700 r, October 12, 1951, following administration of *para*-aminopropriophenone. Killed September 6, 1952, when it was thought that a mild diarrhea observed in this rat might possibly indicate the presence of a tumor. No anatomic cause of the diarrhea was found. A subcutaneous tumor was present on the left flank, measuring 1 cm. in diameter and consisting of round or oval cells with fairly uniform nuclei having finely distributed chromatin and moderate numbers of mitoses. Although the cells formed in some areas a pavement pattern resembling the arrangement of epithelial cells, silver impregnation revealed a complete permeation with fine reticulum fibrils between the individual tumor cells, indicating mesenchymal rather than epithelial origin. The tumor invaded the striated muscle of the abdominal wall and the adipose connective tissue between the intact ducts of a mammary gland. There were no metastases.

Discussion

The incidence of spontaneous tumors in rats varies from strain to strain. No detailed data are available on Sprague-Dawley rats but observations on a limited number of animals indicate that in this as in other strains, mammary fibroadenomas are the most common benign tumors (*4*) and that sarcomas greatly outnumber carcinomas among spontaneous malig-

nant neoplasms. The occurrence of 8 tumors, other than fibroadenomas, in 21 animals that survived for 200 to 500 days after exposure to X radiation appears to be in excess of the incidence of spontaneous tumors reported in several strains of rats (*6*). Moreover, six of the tumors were epithelial in origin. Four of these were adenocarcinomas of the intestinal tract, described only a few times as occurring spontaneously (*7–9*). The renal tumor of the present series is clearly distinct from the moderately common renal tumors with a sarcomatous component (*7*) which are generally classified as either embryonal carcinomas or adenosarcomas (*10*). Purely epithelial renal tumors of tubular origin have been reported but rarely (*7, 11, 12*). An adamantinoma has been described in the rat only once (*13*). Only one published report of a tumor of the central nervous system was traced and this referred to a ganglioneuroma of the optic nerve (*7*). A glioma, closely resembling the one here presented, was observed in a rat exposed to simulated high altitude by Dr. B. Highman in this laboratory. In contrast, the uterine sarcoma found in a non-irradiated litter mate is moderately common (*7*). Thus the spectrum of tumors observed in the irradiated rats deviated markedly from that of spontaneously occurring neoplasms, suggesting that the tumors in the present series were induced by irradiation.

The intestinal tumors are of particular interest since adenomatous proliferations of the epithelium of the small bowel produced by local irradiation were studied in this laboratory in a joint investigation with Bond *et al.* (*14*). The single local irradiation of the gut was applied by a 190 Mev deuteron beam. The doses delivered varied from 1,800 to 2,800 r.e.p., which were comparable in biologic effectiveness to 1,800 to 3,000 r (*14*). At the time of the original report, the longest period of observation following irradiation was seven months. None of the lesions so produced had metastasized, and they could not be classified as potentially malignant or even definitely neoplastic. Similarly irradiated animals have now been observed for 12 months, and in one case masses of mucoid tissue were found scattered over the peritoneal surface of the bowel and the diaphragm. These lesions consisted of aggregates of glandlike cysts of varying size lined with columnar epithelial cells, some of which produced mucus while others had a granular cytoplasm characteristic of Paneth cells. There was no invasion of the muscular wall of the bowel or the diaphragm by the newly formed tissue, and fibrous or granulation tissue separated the mucus-filled cysts from the underlying muscle (fig. 14). Lymph nodes adjacent to one of these lesions were not invaded. In view of the absence of direct invasion or lymph node metastases, a diagnosis of pseudomyxoma peritonei was made. In earlier lesions following local irradiation, ulcerations of the mucosa with fibrous replacement of the entire muscular wall, proliferation of epithelium with formation of diverticula at the edge of the ulcers, and perforations at these sites had been observed. Rupture of pinched-off diverticuli is believed to account for the peritoneal dissemination of proliferating epithelium.

In contrast, two of the lesions of the gut following whole-body irradia-

tion showed invasion of lymphatics, and there was infiltrative growth into ovaries, lungs and adipose tissue. In one of these tumors, the site of origin was clearly established as a localized lesion sharply demarcated from the adjacent normal mucosa. The same localized development was seen in the two polypoid lesions, one of which showed invasion of the muscularis, the other extension into the mesentery. The diagnosis of adenocarcinoma appeared justified in the latter two instances, even in the absence of metastases, since taken together the four intestinal tumors represented an apparent progression from a locally invasive to a metastasizing lesion. There was no evidence that an inflammatory process or proliferation of normal epithelium had preceded these lesions, and it may be inferred that these carcinomas took their origin from a change in the epithelium itself. It is noteworthy that even in the destructively invasive lesions, the normal appearance of the intestinal epithelium, including the differentiation into goblet and Paneth cells, was occasionally faithfully reproduced, a feature of intestinal carcinoma of rats noted by Dunn (*15*).

It appears warranted to conclude that the histogenesis of the malignant tumors of the intestine following whole-body irradiation differs from that of the adenomatous lesions following local irradiation in that the former appears to be initially proliferative and the latter secondary to ulceration and dislocation of epithelium into scar tissue.

The renal carcinoma of the present experiments may be similarly contrasted with a case of epithelial proliferation in a locally irradiated kidney. In a series of unpublished experiments Bond irradiated the spinal cord of rats by a single tangential exposure of the back of the rat to 3,000 r.e.p. of the 190 Mev deuteron beam. In most instances, one or both kidneys were in the field of irradiation. All of these animals developed necrosis of the spinal cord and most of them succumbed within a few months. Vascular changes and predominantly tubular damage were seen in all irradiated kidneys. The kidney of the single rat that survived irradiation for 12 months showed, in addition, epithelial proliferation apparently originating from the tubular epithelium (fig. 15). High-power views of individual fields were strikingly similar to the renal carcinoma here described. However, there was neither invasion of perirenal tissues nor distant metastases.

The findings in both the kidney and intestinal lesions point up the difficulties encountered in diagnosing proliferative epithelial lesions in the rat as malignant. It is well known that the intestinal mucosa of the rat is particularly prone to proliferation extending into preformed planes of separation between muscle bundles, thus simulating invasion (*9*). The present observations serve to re-emphasize that only the progression to organ invasion or metastasis can establish the diagnosis of malignancy in rat tumors with certainty, at least at the present state of our knowledge.

Protection from the immediate lethal effects of single whole-body irradiation does not ensure a restitution to the pre-irradiation state. Rats protected by anoxia have been shown to have a shortened life span (*16*), and the present experiment indicates that this is also true of rats

given *para*-aminopropriophenone or glutathione before exposure to X ray or joined in parabiosis following irradiation.

The 30-day LD_{99} for Sprague-Dawley rats of weights and ages similar to those employed in the present experiment has been previously determined to be 660 ± 30 (*3*); 700 r may therefore be considered a close to uniformly lethal dose and the doses of 800 to 1,000 r as invariably fatal. Only treated animals survive these larger doses. They cannot, therefore, be directly compared with untreated irradiated controls. A comparison is possible, however, between animals exposed to these larger doses following administration of *para*-aminopropriophenone and similarly exposed animals protected by parabiosis or glutathione. Such experiments may demonstrate some differences in the incidence of late effects in animals subjected to the various treatments and possibly some measure of protection by one or the other of the treatments against long-term radiation effects, such as the induction of tumors.

Summary

1) Eight malignant tumors occurred in 21 rats protected from lethal doses of irradiation either by pretreatment with glutathione or *para*-aminopropriophenone, or by parabiosis before or after irradiation. Six of the eight tumors were epithelial in origin, and all were locally invasive. Three metastasized.

2) The high incidence of tumors in this small group of animals, and the incidence of tumor types that seldom occur spontaneously, indicate that these neoplasms were induced by whole-body irradiation.

3) The histogenesis of four adenocarcinomas of the intestinal tract in these animals differed from that of previously described proliferative epithelial lesions of the mucosa following single local irradiation in that they were not preceded by ulceration and scarring, but seemed to arise *de novo* from an apparently intact mucosa.

The authors are indebted to Dr. V. P. Bond and his associates at the Naval Radiological Defense Laboratory, San Francisco, for their permission to use material from a joint study on the effects of local deuteron irradiation in the present paper.

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PLATE 15

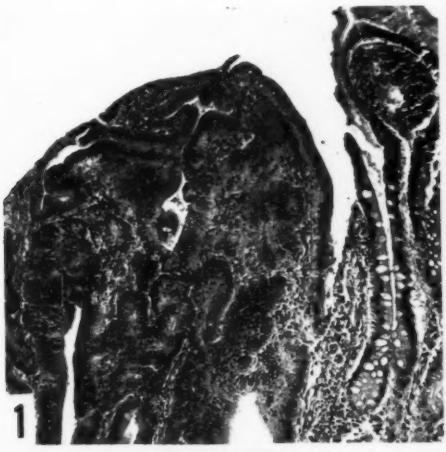
FIGURE 1.—Path. No. 57379. Edge of adenocarcinoma of jejunum in irradiated rat protected by parabiosis. Abrupt change to normal mucosa. × 80

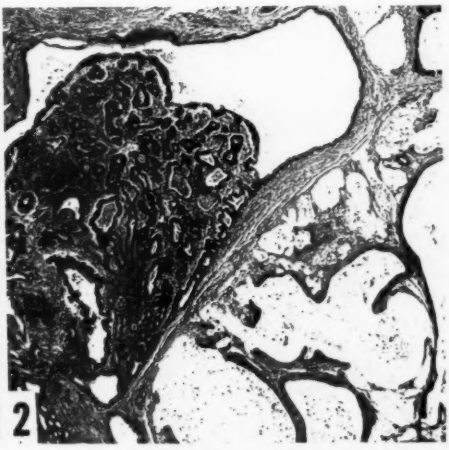
FIGURE 2.—Same tumor as in figure 1. Invasion of mesentery by both papillary and cystic growth, the latter resembling mucus-producing adenomatous lesions produced by local irradiation. × 35

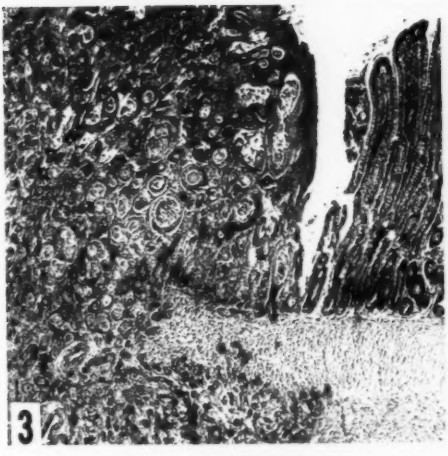
FIGURE 3.—Path. No. 57563. Adenocarcinoma of ileum in irradiated rat protected by parabiosis. Edge of primary lesion. × 30

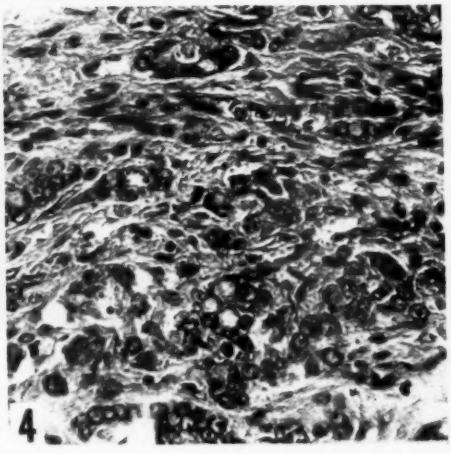
FIGURE 4.—Same tumor as in figure 3. Metastasis. × 350

FIGURE 5.—Path. No. 60526. Edge of polyp of colon in irradiated rat protected by parabiosis. Invasion of stalk. × 30









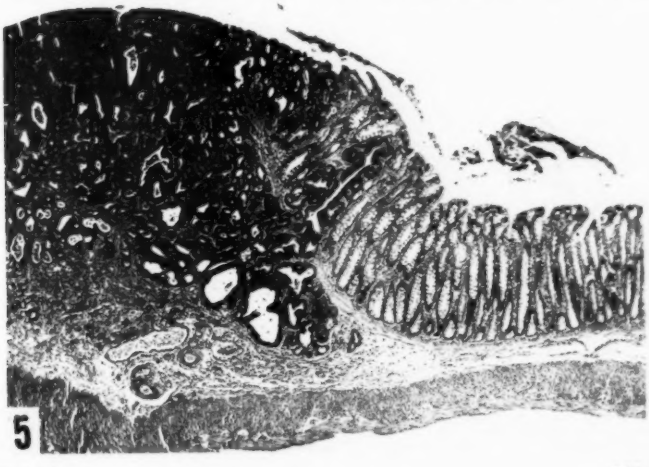


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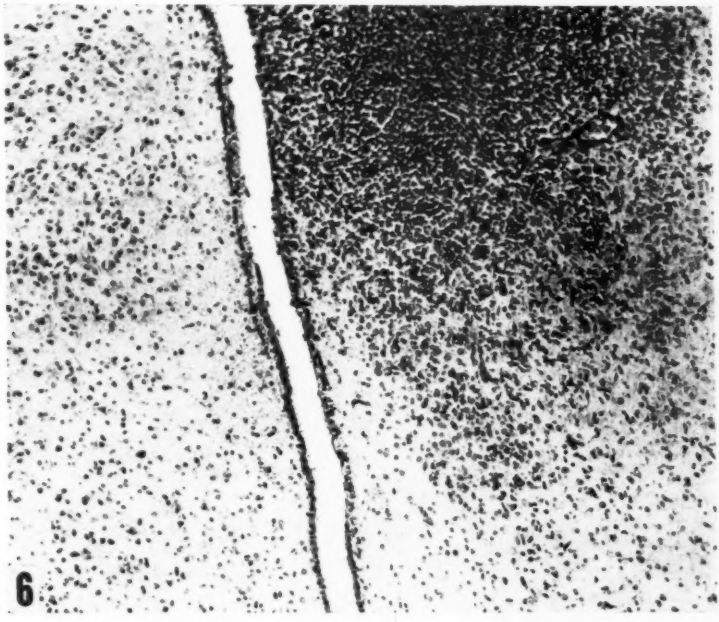
PLATE 16

FIGURE 6.—Path. No. 59169. Glioma in irradiated rat protected by parabiosis. × 30

FIGURE 7.—Same tumor as in figure 6. Edge of glioma with tumor cells spreading into normal brain tissue. × 400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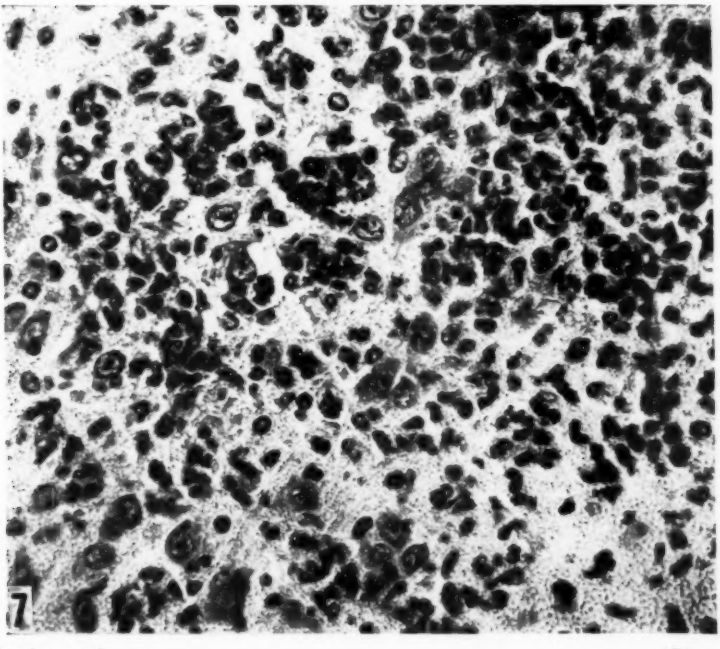
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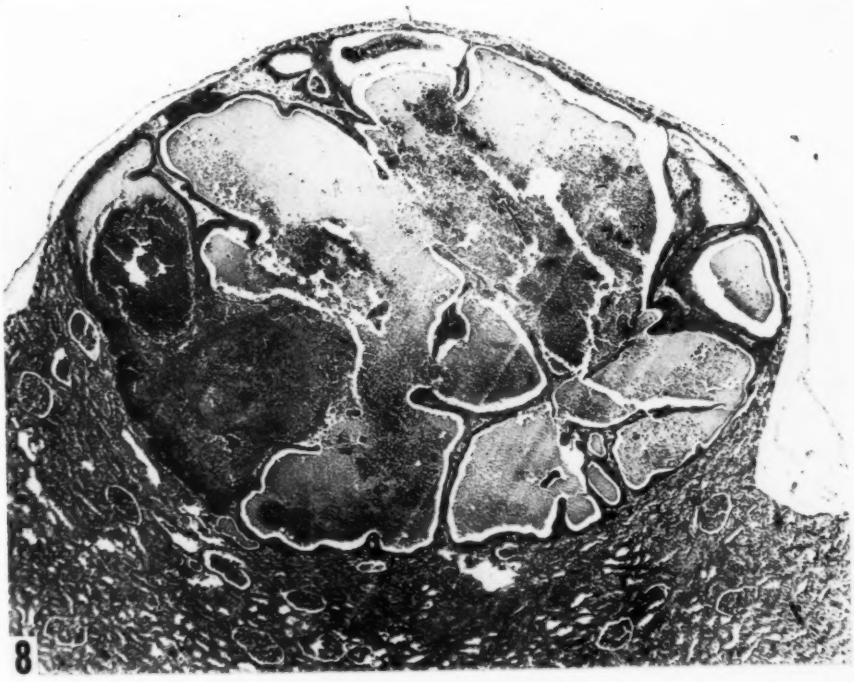
PLATE 17

FIGURE 8.—Path. No. 59170. Renal carcinoma of irradiated rat protected by *para*-aminopropriophenone. Cystic lesion. × 45

FIGURE 9.—Same tumor as in figure 8. Metastasis with solid growth and invasion of glomerulus. × 90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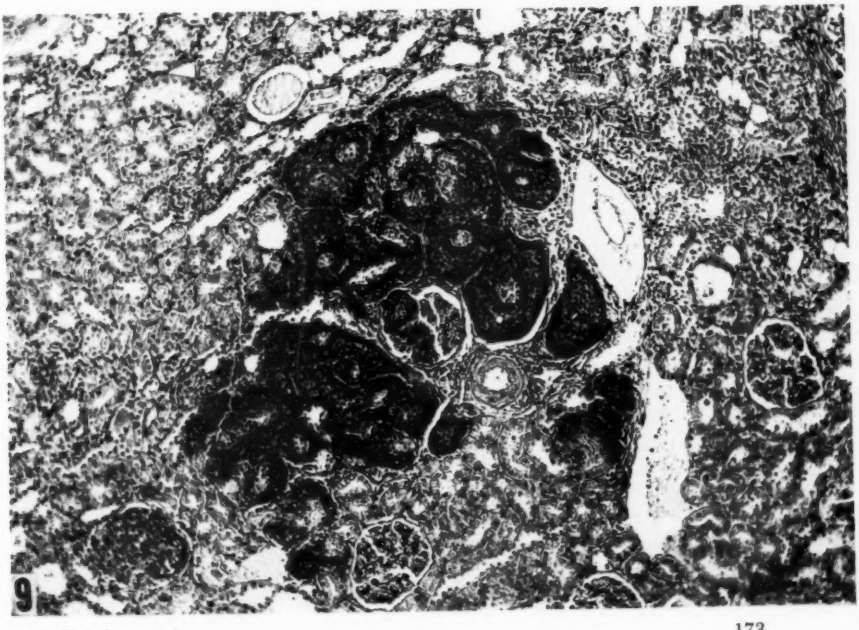
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PLATE 18

FIGURE 10.—Same tumor as in figure 8. Pulmonary metastasis of renal carcinoma. Superficial keratinization. Invasion of bronchus. × 80

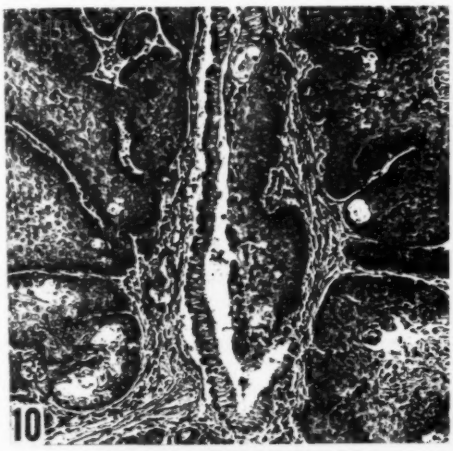
FIGURE 11.—Same case as figure 8. Metastasis in cervical lymph node. × 85

FIGURE 12.—Path. No. 57380. Adamantinoma of irradiated rat protected by *para*-aminopropriophenone. × 85

FIGURE 13.—Same tumor as in figure 12. Secretory granules in palisaded tumor cells adjacent to cement-like material. × 500

FIGURE 14.—Adenomatous nodule on diaphragm (pseudomyxoma peritonei) following local deuteron irradiation of gut. × 35

FIGURE 15.—Hyperplasia of tubular epithelium following local deuteron irradiation of kidney. × 100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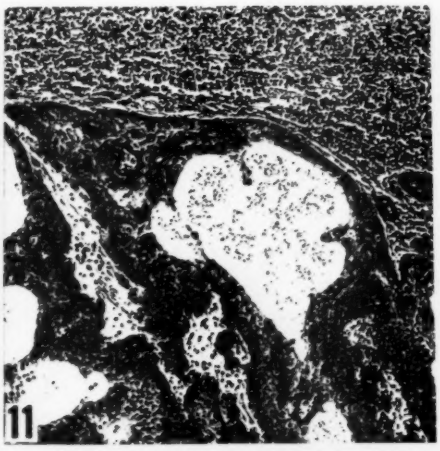
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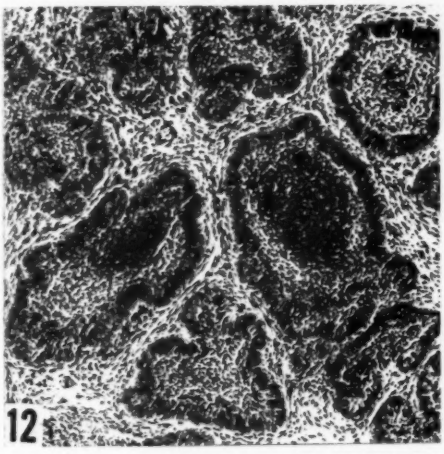
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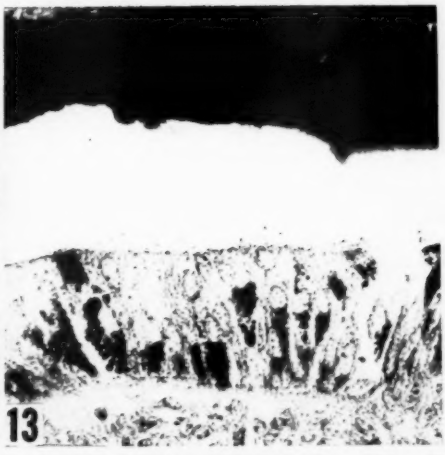
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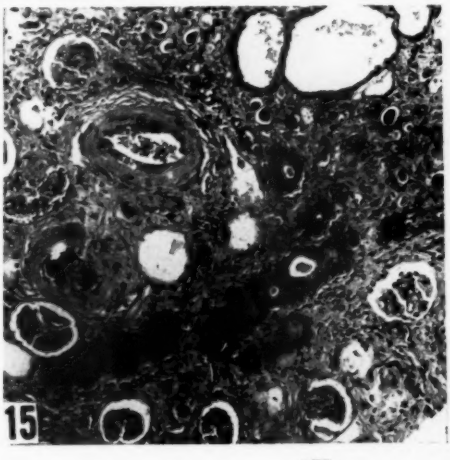
15

Enzyme Changes Induced in Normal and Malignant Tissues with Chemical Agents. II. Effect of Various Compounds on the Cytochrome Oxidase Activity of Transplanted Tumors [1, 2, 3]

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Laboratory of Chemical Pharmacology, National Cancer Institute,[5] Bethesda, Md.

In a previous paper (*2*) we reported that compounds isolated from podophyllin, which were highly active in producing hemorrhage and necrosis in Sarcoma 37, caused a marked drop in the cytochrome oxidase activity in the tumor within a period of 6 to 8 hours after a single subcutaneous injection. A relatively smaller decrease in enzyme levels was observed in normal tissues of mice, with or without tumors, following injection of even larger doses of these compounds. Only slight changes were observed following incubation *in vitro* of homogenates of tumor or of normal tissues with these agents.

This paper presents data on the effect upon cytochrome oxidase levels in normal and tumor tissues of a variety of chemical agents potent in damaging Sarcoma 37. Data are also presented on the enzyme changes produced in several other tumors that are damaged by these agents.

Materials and Methods

The *alpha*-peltatin, *beta*-peltatin, and podophyllotoxin employed were isolated from podophyllin (*3*). Their potency in damaging Sarcoma 37, and other tumors, has been described (*4, 5*). Derivatives of colchicine, arsenic, antimony, phenazine, and quinoxaline were either prepared in this Laboratory or obtained from other sources. Studies on the potency of some of these compounds in damaging Sarcoma 37 have also been published from this Laboratory (*6, 7*). The compounds reported in this paper, with their National Cancer Institute accession numbers, are listed on the following page.

[1] Received for publication April 22, 1953.

[2] The first paper in this series (*2*) was entitled "Effect of podophyllotoxin, *alpha*-peltatin and *beta*-peltatin on the cytochrome oxidase activity of Sarcoma 37."

[3] Presented in part at the Annual Meeting of the American Association for Cancer Research, 1950 (*1*).

[4] Visiting Scientist, National Cancer Institute, Bethesda, Md.

[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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NCI No.	*Compound*
1v	Colchicine
192	Mapharsen
237	*p*-phenetedine antimonyl tartrate
368	N-acetyliodocolchinol methyl ether
829	5,5′-Diamino-2,2′-dimethylarsenobenzene-N,N′-dimethylenesulfonate disodium salt
1037	N-acetyliodocolchinol
1040	Colchiceine
1073	Podophyllotoxin
1074	*alpha*-Peltatin
1136	Trimethylcolchicinic acid methyl ether *d*-tartrate
1778	*o*-Aminophenol antimonyl tartrate
1839	*beta*-Peltatin
1894	N-acetylcolchinol
1895	N-acetylcolchinol methyl ether
1921	N-methylquinoxalinium iodide
2285	2-Chloro-8-methoxy-5-methylphenazinium methyl sulfate
2561	2-Bromo-8-ethoxy-5-methylphenazinium methyl sulfate

The solvents employed were distilled water, dilute sodium hydroxide, or a 30 percent aqueous solution of propylene glycol; in some instances, the compounds were dispersed in olive oil.

The transplanted tumors used were: *a*) six-day-old intramuscular implants of Sarcoma 37 in CAF_1 mice; *b*) twelve-day-old intramuscular implants of Lymphoma 1 and of Lymphoma 2 (*8*); *c*) ten-day-old intramuscular implants of Mammary Adenocarcinoma C3HBA in C3H mice (9); *d*) six-day-old implants of Leukemia 1210 in DBA mice (*10*); and *e*) eighteen-day-old implants of Melanoma S-91 in DBA mice.

The tumor-bearing animals were given a single subcutaneous injection; at least four animals were killed at each time interval from 2 to 48 hours after injection. The effect of the compounds on the tumors and organs was observed grossly and microscopically, and the cytochrome oxidase activity of homogenates of these tissues was determined. A group consisting of at least four untreated tumor-bearing mice for each 24-hour interval was employed for comparison with the effects of the compounds on tumors and organs from the treated animals. The techniques for the preparation of homogenates were similar to those employed previously (*2*).

The method of determining the cytochrome oxidase activity was similar to that described previously (*2*). Usually a 1 to 10 homogenate was employed; in a few instances a 1 to 100 dilution was used. The rate of autoxidation was measured by extrapolating to zero tissue concentration, following the procedure of Schneider and Potter (*11*). The Q_{O_2} values in these determinations were considerably greater than those obtained with a 1 to 10 dilution. As stated in a previous publication (*2*), a number of experiments were performed using both 1 and 10 percent homogenates. In all cases the relative changes in enzyme activity were similar, whether determined in a 1 percent or in a 10 percent tissue suspension.

Results

Effect of Different Types of Chemical Agents on Cytochrome Oxidase Activity in Sarcoma 37

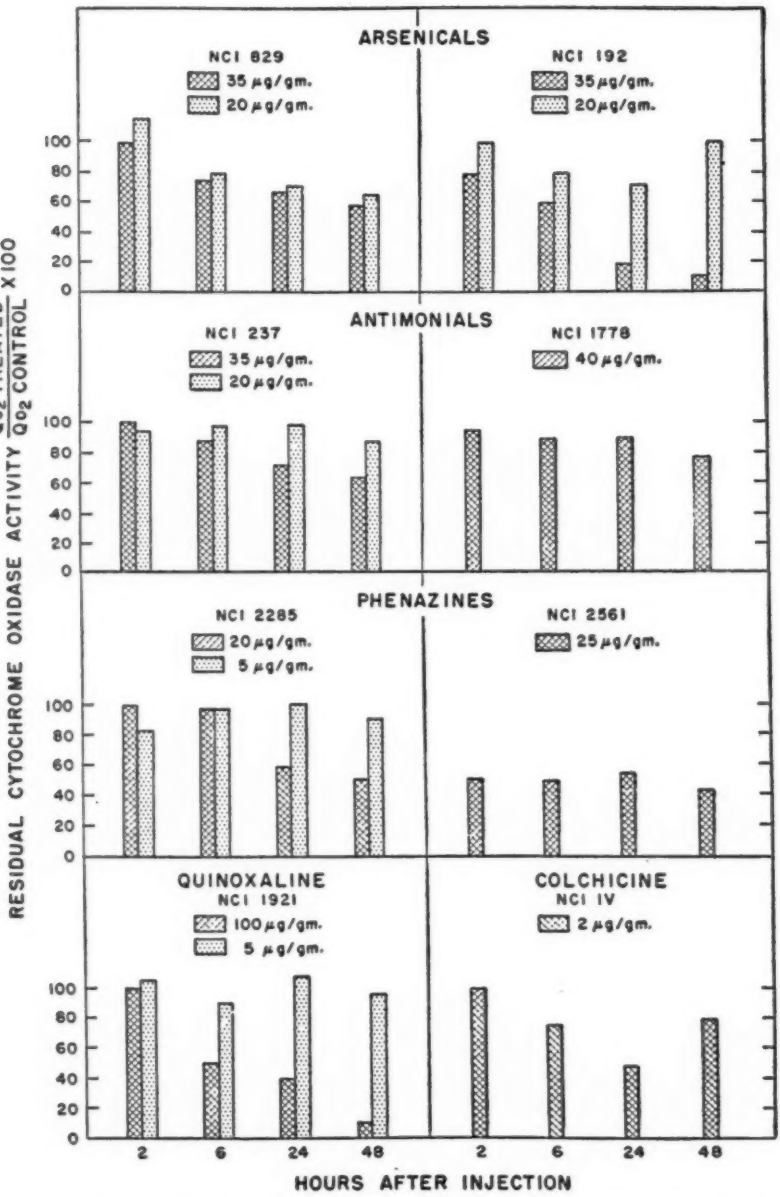
Several classes of compounds potent in damaging Sarcoma 37 were selected for determining the effect on the cytochrome oxidase activity in tumor and liver homogenates after a single subcutaneous injection into mice bearing six-day-old implants of Sarcoma 37. Two dose levels were usually employed. The higher dose was in the range of the maximum tolerated dose (MTD); the lower dose was at or slightly below the minimum effective dose (MED). With most of the compounds the range between these doses was small. The compounds employed were 2 arsenicals, 2 antimonials, 2 phenazines, 1 quinoxaline, and 4 colchicines. Summaries of the data are presented in text-figures 1 and 2. At the higher dose levels grossly evident damage was induced in tumors, and there was a significant drop in the residual cytochrome oxidase level to 60 percent or less of the control value within 24 hours after injection. In the case of the two antimonials the drop appeared to be small. In the case of one of these (NCI-1778), neither induced damage nor any marked reduction in the enzyme activity was produced. Since induced damage had been observed with this antimonial some time earlier at this dose level, it is possible that deterioration of the compound such as had been observed with arsenicals (*6*) had occurred here too. The damage induced in the tumors at the lower dose level (MED) was generally minimal, and the reduction in enzyme activity was much smaller than that observed at the higher dose level.

The drop in cytochrome oxidase activity was very rapid with one of the phenazinium compounds, NCI-2561. The enzyme activity in the tumor tissue dropped to less than 50 percent of that of the controls in 2 hours.

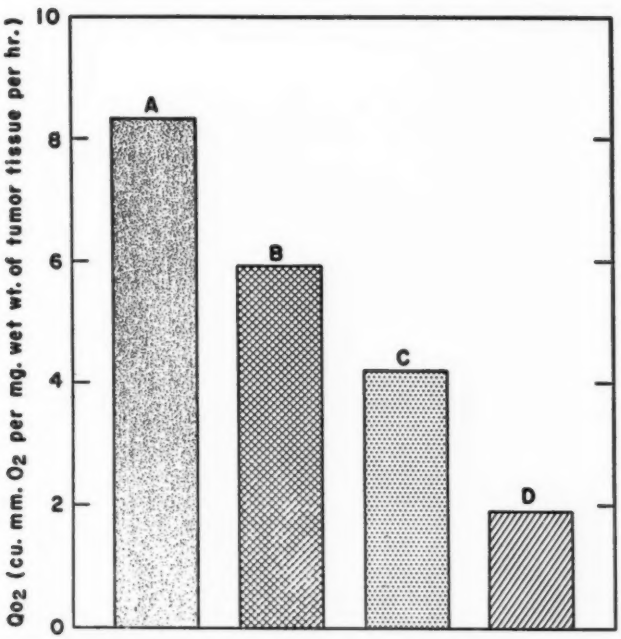
In all instances the cytochrome oxidase activity in liver homogenates from the same animals was determined. Only small changes were observed in the liver. In most instances, over 90 percent of the activity of control tissues remained; in all instances, over 80 percent of the activity was still present.

Effect of Chemical Agents on Cytochrome Oxidase Activity of a Variety of Mouse Tumors

Lymphoma 2.—Text-figure 3 summarizes the data obtained on the cytochrome oxidase activity in homogenates of tumor, spleen, lymph nodes, and liver of mice bearing Lymphoma 2 following a single injection of podophyllotoxin (NCI-1073), *alpha*-peltatin (NCI-1074), or *beta*-peltatin (NCI-1839). The results were similar with all three compounds. Reduction of enzyme activity to about half of that in tissues of untreated control animals occurred in tumor tissue, spleen and in lymph nodes.



TEXT-FIGURE 1.—Reduction in cytochrome oxidase activity, in homogenates of mouse Sarcoma 37, from mice 2, 6, 24, and 48 hours after a single subcutaneous injection of various chemical agents.

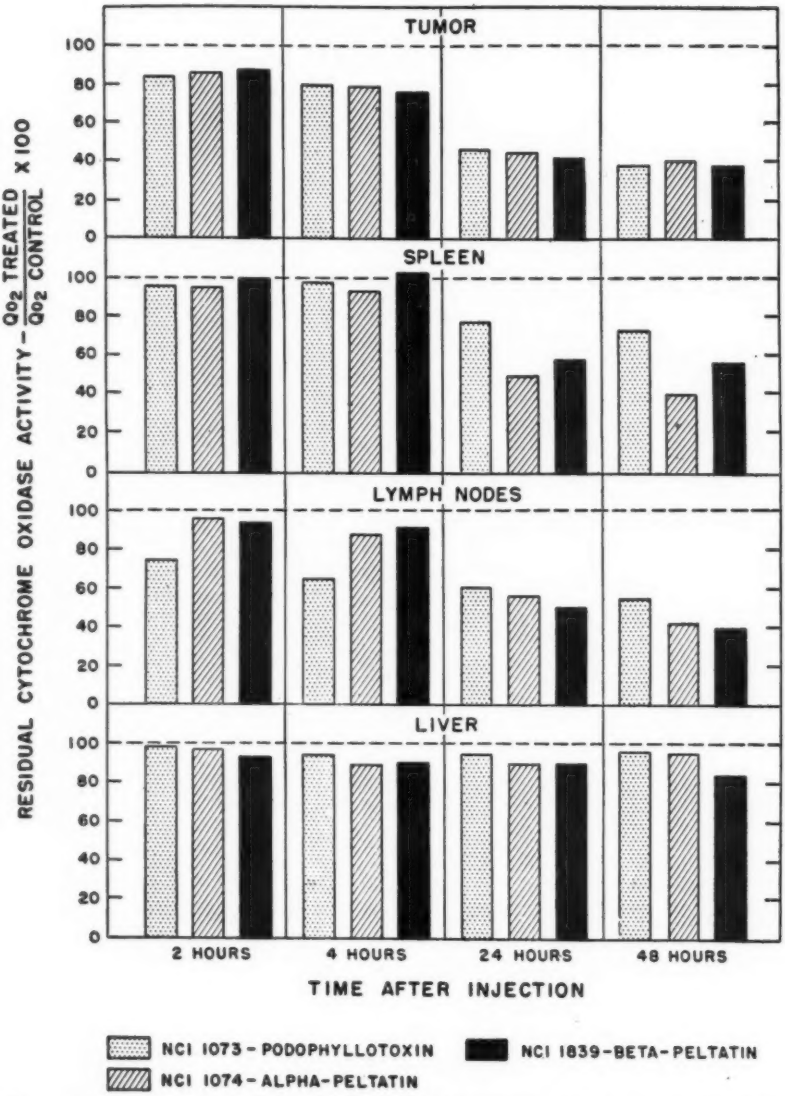


TEXT-FIGURE 2.—Reduction in cytochrome oxidase activity, in homogenates of mouse Sarcoma 37, from mice 24 hours after a single subcutaneous injection of one of 3 colchicine derivatives.

A—Controls
B—N-Acetyliodocolchinol (NCI-1037) (250 μg./gm.)
C—N-Acetyliodocolchinol methyl ether (NCI-368) (300 μg./gm.)
D—Trimethylcolchicinic acid methyl ether *d*-tartrate (NCI-1136) (100 μg./gm.)

The cytochrome oxidase activity in liver showed little change. It is likely that the marked drop in enzyme activity in spleen and lymph nodes, in contrast to the smaller change in liver, may be due to the fact that the former organs are virtually replaced by infiltrated tumor tissue (*8*), whereas the infiltration in liver is less extensive.

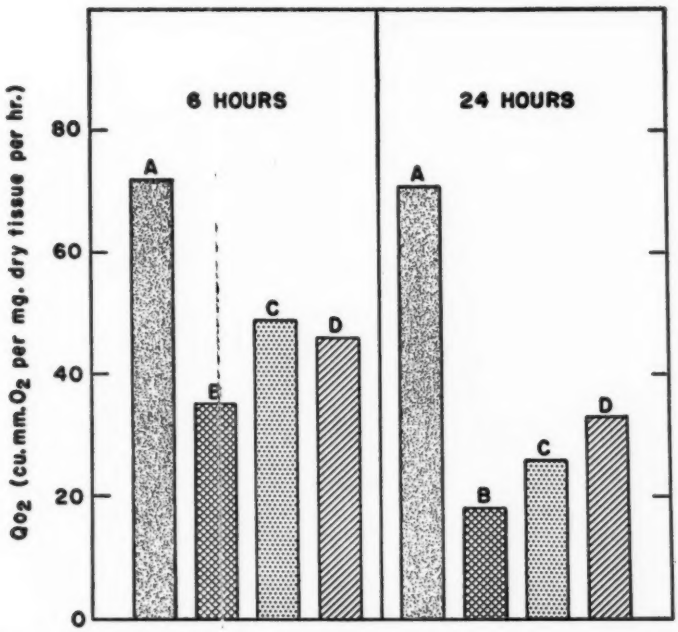
Text-figure 4 illustrates results obtained with the same tumor (Lymphoma 2) following a single injection of 3 derivatives of colchicine: trimethylcolchicinic acid methyl ether *d*-tartrate (NCI-1136), N-acetylcolchinol (NCI-1894), or N-acetylcolchinol methyl ether (NCI-1895). Six hours after injection, only about one-half of the cytochrome oxidase activity remained. By 24 hours there remained only about one-fourth of the enzyme activity observed in tumors from untreated controls. The pattern observed in spleen, lymph nodes, and liver was similar to that illustrated in text-figure 3 for the components of podophyllin. Several other derivatives of colchicine, including colchicine (NCI-1v), colchiceine (NCI-1040), N-acetyliodocolchinol (NCI-1037) and its methyl ether (NCI-368), gave comparable effects. All the colchicine compounds described here were active in damaging Sarcoma 37 as well as Lymphoma 2.



TEXT-FIGURE 3.—Reduction in cytochrome oxidase activity in homogenates of Lymphoma 2, spleen, lymph nodes, and liver from mice after a single subcutaneous injection of 20 μg./gm. of podophyllotoxin (NCI-1073), *alpha*-peltatin (NCI-1074), or *beta*-peltatin (NCI-1839). Control values for enzyme activity for a 10% tissue homogenate (cu. mm. O_2/mg. dry tissue/hour):

Lymphoma 2.....65.4–72.1
Spleen.....72.3–79.5
Lymph nodes.....47.5–56.8
Liver.....110.0–120.3

Since some of these organs were infiltrated with tumor, their values should not be compared to those obtained with normal organs.



TEXT-FIGURE 4.—Reduction in cytochrome oxidase activity in homogenates of Lymphoma 2 from mice 6 and 24 hours after a single injection of one of 3 colchicine derivatives. A 10% tissue homogenate was employed in these determinations.

A—Control tissue

B—Trimethylcolchicinic acid methyl ether *d*-tartrate (NCI–1136) (60 μg./gm.)

C—N-Acetylcolchinol (NCI-1894) (100 μg./gm.)

D—N-Acetylcolchinol methyl ether (NCI-1895) (15 μg./gm.)

Leukemia 1210.—A decrease in cytochrome oxidase activity in this tumor to 12 percent of the control values occurred 24 hours after a single injection of 20 μg. per gm. of podophyllotoxin (NCI-1073) (table 1). A smaller decrease (to 67 percent) was produced by a dose of 2 μg. per gm. of colchicine (NCI-1v). These doses represent approximately two-thirds of the maximum tolerated dose of each drug; the dose of podophyllotoxin was well above the minimum effective dose, whereas the dose of colchicine was very close to the MED. With each compound, little change occurred in the enzyme activity of liver homogenates from the same animals.

Lymphoma 1.—Text-figure 5 shows a marked drop in the cytochrome oxidase activity in homogenates of Lymphoma 1 following an injection of 20 μg. per gm. of either podophyllotoxin (NCI-1073) or *beta*-peltatin (NCI-1839). In 3 hours only about one-third of the enzyme activity found in controls remained in tumors from mice injected with the *beta*-compound. By 24 hours there was virtually no enzyme activity in the tumor. By 48 hours, there seemed to be a rather marked increase in

TABLE 1.—In vivo *effect of podophyllotoxin and of colchicine on cytochrome oxidase activity of Leukemia 1210*

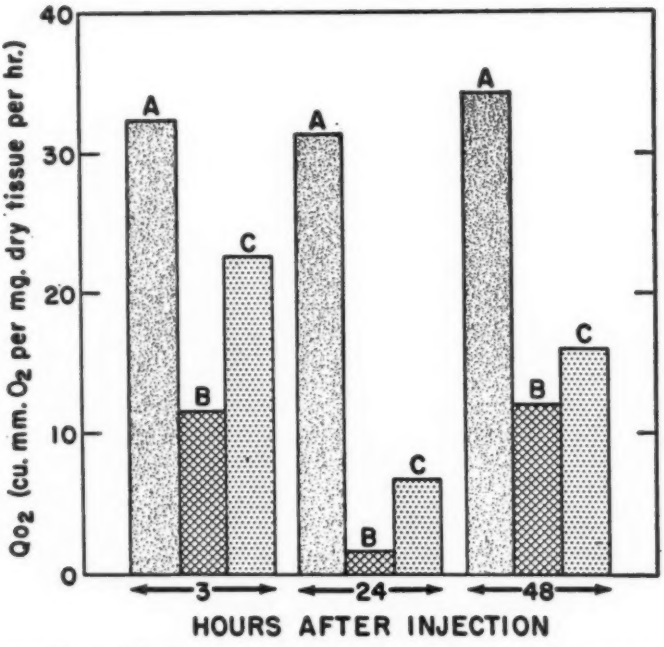
Time after injection (*hours*)	Residual activity*			
	Podophyllotoxin†		Colchicine‡	
	Tumor (*percent*)	Liver (*percent*)	Tumor (*percent*)	Liver (*percent*)
2	87	111	87	107
4	84	102	96	101
24	12	101	67	93

* Values expressed as $\frac{Q_{O_2}\ \text{treated}}{Q_{O_2}\ \text{control}} \times 100$.

Q_{O_2} (cu. mm. O_2 per mg. dry tissue per hr.) control values—Leukemia 1210: 150.0–169.3; liver: 331.0–332.5. 1% tissue homogenate was used in these determinations.

† 20 μg./gm. injected subcutaneously.

‡ 2 μg./gm. injected subcutaneously.



TEXT-FIGURE 5.—Reduction in cytochrome oxidase activity in homogenates of Lymphoma 1 from mice 3, 24, and 48 hours after a single injection of 20 μg./gm. of *beta*-peltatin or of podophyllotoxin. A 10% tissue homogenate was used in these determinations.

A—Control
B—*beta*-Peltatin (NCI-1839)
C—Podophyllotoxin (NCI-1073)

activity over the 24-hour period, suggesting a rapid recovery in the tumors. This marked increase after 48 hours or longer was not observed in any of the other tumors described here, or in Sarcoma 37 (*2*).

Adenocarcinoma C3HBA and Melanoma S-91.—Tables 2 and 3 show a marked diminution in enzyme activity in homogenates of both these tumors following an injection of either podophyllotoxin (NCI-1073) or of colchicine (NCI-1v), with much smaller changes occurring in livers of the same animals. Although initially the cytochrome oxidase activity was relatively high in melanoma, a marked decrease in enzyme activity

TABLE 2.—In vivo *effect of podophyllotoxin and of colchicine on the cytochrome oxidase of Mammary Adenocarcinoma C3HBA*

Time after injection (*hours*)	Residual activity*			
	Podophyllotoxin†		Colchicine‡	
	Tumor (*percent*)	Liver (*percent*)	Tumor (*percent*)	Liver (*percent*)
2	88	88	75	93
4	90	91	71	93
24	68	86	17	92

* Values expressed as $\frac{Q_{O_2}\ \text{treated}}{Q_{O_2}\ \text{control}} \times 100$.

Q_{O_2} (cu. mm. O_2 per mg. dry tissue per hr.) control values—tumor: 106.7–112.3; liver: 371.5–374.0. 1% tissue homogenate was used in these determinations.

† 20 μg./gm. injected subcutaneously.

‡ 3 μg./gm. injected subcutaneously.

TABLE 3.—In vivo *effect of podophyllotoxin and of colchicine on cytochrome oxidase activity of Melanoma S-91*

Time after injection (*hours*)	Residual activity*			
	Podophyllotoxin†		Colchicine‡	
	Tumor (*percent*)	Liver (*percent*)	Tumor (*percent*)	Liver (*percent*)
2	100	103	96	93
4	80	100	99	98
6	49	93	102	100
24	29	90	56	86
48	22	93	44	95

* Values expressed as $\frac{Q_{O_2}\ \text{treated}}{Q_{O_2}\ \text{control}} \times 100$.

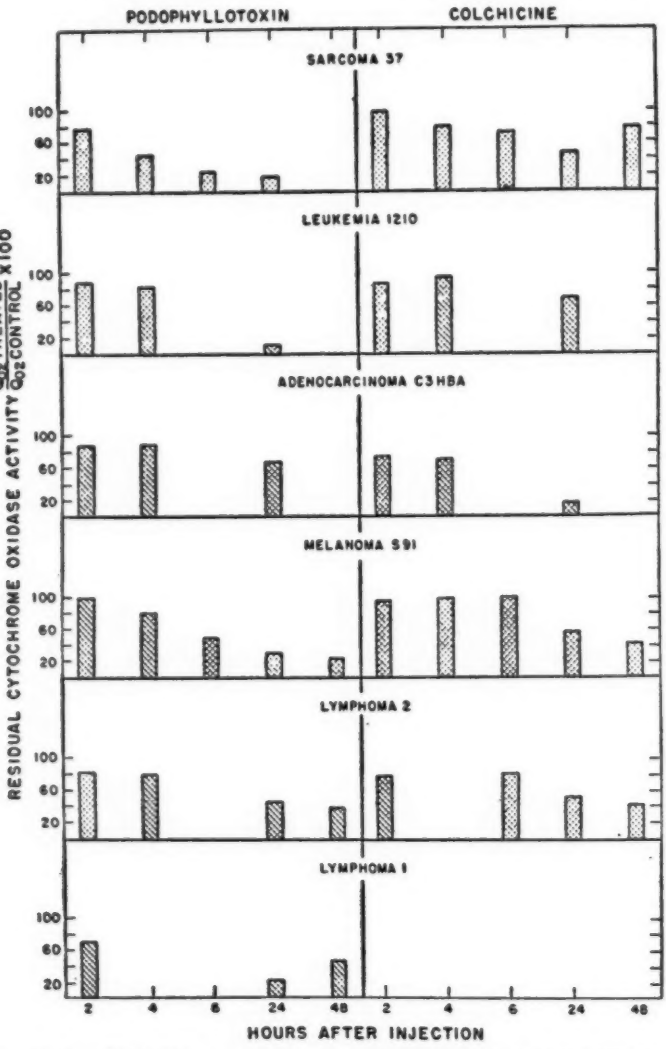
Q_{O_2} (cu. mm. O_2 per mg. dry wt. tissue per hour) control values—tumor: 229.6–248.6; liver: 334.6–357.7. 1% tissue homogenate was used in these determinations.

† 20 μg./gm. injected subcutaneously.

‡ 2 μg./gm. injected subcutaneously.

occurred similar to that observed in other tumors having a much lower enzyme activity initially. The enzyme activity in this melanoma is higher than in many normal tissues and is in the same range as that in liver, which has high cytochrome oxidase activity.

Text-figure 6 summarizes the effects produced by podophyllotoxin and colchicine on the cytochrome oxidase activity in all six transplantable tumors, including Sarcoma 37, that were employed in these investigations.



TEXT-FIGURE 6.—Reduction in cytochrome oxidase activity in homogenates of six tumors following a single injection of either 20 μg./gm. of podophyllotoxin (NCI-1073) or of 2–3 μg./gm. of colchicine (NCI-1v).

The same qualitative changes were observed in all these tumors which were affected by these two compounds.

Discussion

The results reported in this paper extend our previous findings (*2*) on the effect of compounds isolated from podophyllin on the cytochrome oxidase activity of Sarcoma 37. Five different chemical classes of compounds, all capable of inducing grossly evident damage in Sarcoma 37, produced a marked decrease in the cytochrome oxidase activity in homogenates of Sarcoma 37. The pattern of change in enzyme activity was similar to that observed with podophyllotoxin and peltatins. Only a small change in the cytochrome oxidase activity of liver homogenates was concomittantly observed with these chemical agents.

Reduction in cytochrome oxidase activity also occurred after the injection of some of these chemical agents into animals bearing different types of tumors, *viz.* a local lymphoma, a metastasizing lymphoma, an acute lymphoid leukemia, a mammary adenocarcinoma, and a melanoma.

Extensive tumor infiltration occurred in spleen and lymph nodes of mice bearing the metastasizing lymphoma (Lymphoma 2), but occurred only to a limited extent in liver. The decrease in cytochrome oxidase activity in these infiltrated organs (spleen and lymph nodes) was much larger than that occurring in the liver of the same animals or in the spleens and lymph nodes of mice bearing a non-metastasizing tumor (Sarcoma 37) or no tumor (*2*). The drop in enzyme activity of spleen and lymph nodes in mice bearing Lymphoma 2 is probably a reflection of the damage induced in the tumor tissue in these organs by the chemical agents. Even in the case of the melanoma, which has a considerably higher cytochrome oxidase activity than many of the tumors investigated, the relative drop in enzyme activity was as marked as in tumors of lower enzyme activity, and was greater than the drop in activity of normal tissues of higher, equivalent, or lower enzyme activity.

At the present time it is not possible to determine any common factor in these different chemical agents that is responsible for the drop in cytochrome oxidase activity in the tumors other than their ability to produce morphologic damage in them. It is not likely that any one specific property (such as binding of sulfhydryl groups by arsenicals) would be common to all these compounds. The mechanism by which these agents all produce damage in the tumors and a drop in cytochrome oxidase activity requires further elucidation.

Summary

1) A single subcutaneous injection of tumor-damaging agents (2 arsenicals, 2 antimonials, 2 phenazines, 1 quinoxaline, and 4 colchicines) in mice bearing Sarcoma 37 produced a marked drop in cytochrome oxidase activity in tumor tissue homogenates within 24 hours after injection.

2) A similar reduction in the cytochrome oxidase activity of tumor

tissue homogenates was also observed when some of these agents were injected into animals bearing Lymphoma 1, Lymphoma 2, Leukemia 1210, Mammary Adenocarcinoma C3HBA, or Melanoma S-91.

3) In the case of Lymphoma 2, spleen and lymph nodes which were heavily infiltrated with tumor showed a similar marked drop in enzyme activity. Liver, whether from animals bearing this tumor or other tumors, was little affected.

References

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The Effect of Ultracentrifugation and Hyaluronidase on the Filtrability of Chick-Embryo Extract for Tissue Culture [1, 2]

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Survival or proliferation of a tissue *in vitro* for periods of time longer than a few hours requires the presence of nutrient materials in the culture medium. At the present state of our knowledge, rapid cell proliferation for extended periods of time demands that the nutrient medium include an extract from embryonic tissue. This extract has been prepared by briefly extracting finely minced tissue with one or more volumes of a balanced physiological saline such as that of Ringer, Tyrode, Gey, or Earle, and then separating the active fluid by centrifugation. Principal sources of embryonic tissue have been the 9- to 10-day-old chick embryo and the young beef embryo freshly removed from the gravid uterus.

In the absence of a satisfactory method for routine sterilization of embryo extract it has been necessary to use aseptic technique for each step in the removal of the embryo from the egg or the gravid uterus and in subsequent preparation and handling of the embryo extract. This necessity has accounted for much labor and expense in the maintenance of tissue cultures, and has severely limited chemical manipulation and fractionation of the extract. Further, even though the solution was clarified or frozen, the usual methods of preparation have failed to eliminate the hazard of contamination of the cultured cell strain by viable tissue cells carried over in the extract (*1*).

Many attempts have been made, both in this laboratory and others, to sterilize embryo extract by filtration through various types of bacteriological filter candles and membranes. Either the candle or membrane has rapidly clogged, or filtration has been slow and the filtrate has been low in protein and in ability to induce cell proliferation.

The purpose of the present investigation has been to work out a practical method by which a highly potent extract from 9-day chick embryos could be routinely prepared and sterilized by filtration in large volumes

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[2] This paper was presented in preliminary form at the annual meeting of the Tissue Culture Association in Detroit, Michigan, March 20, 1951.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

for use in tissue culture. In the development and validation of this procedure it was necessary: 1) to obtain and more accurately standardize a silicate-type filter that would prove useful in routine sterilization of embryo extract; 2) to evaluate more quantitatively the actual changes previously observed in the embryo extract during filtration; 3) to define more accurately and to eliminate factors that tend to clog such filters and reduce the activity of the filtrate.

This investigation has permitted the selection and standardization of filter membranes more satisfactory for such work. Factors interfering with filtration have been eliminated to such an extent that it is now possible to filter routinely the extract from chicks of nine days' incubation. The resultant filtrate is of high activity in maintaining cell proliferation.

General Materials and Methods

Preparation of chick-embryo extract

Chick-embryo extract was prepared by methods previously described (*2*). Embryos that had been incubated 9 or 10 days were minced by forcing the tissue through a 24-mesh, 18-8 stainless steel wire gauze held in the lower end of the barrel of a 50-ml. Luer syringe.[4] In most instances Earle's saline (*2*) was added in approximately equal volume to make up a 1:1 embryo extract. The mixture was agitated briefly by sucking in and out of a 50-ml. widemouthed pipette, and was then centrifuged at 2,300 r.p.m. (1,600 × g) for 30 minutes at 4° C. in a refrigerated International No. 3 centrifuge. The supernatant was pipetted into heavy-walled pyrex centrifuge tubes, which were placed in powdered CO_2 ice. The frozen supernatant was then either thawed for immediate experimental use or was stored at −10° C. for later use.

In the preliminary experiments on filtrability, in which the solution was not actually used on the cultures and filtrability was the chief index of a satisfactory solution, no aseptic precautions were necessary, except insofar as it was necessary to see that no bacterial growth supervened before chemical analyses were carried out. In later studies, when an aseptic solution was necessary for tests on cultures, precautions were taken to insure that solutions were kept chilled preceding filtration and that operations were so expedited that any complication from bacterial infection of the solutions was eliminated. During and following filtration of such solutions rigid asepsis was preserved.

Unless otherwise designated, experimental work was carried out on embryo extract within 8 days after preparation. Sterile embryo extract accumulated as surplus from routine laboratory work had been pooled and stored at −10° C. for periods up to 12 months. Extracts taken from this surplus and used in certain experiments will be designated as stored extract.

Ultracentrifugation

High-speed centrifugation was carried out in two different ultracentrifuges. In experiments 3, 4, and 6 to 10 a Spinco Model E ultracentrifuge[5] was operated[6] with a Type D Preparative Rotor having a capacity of 456 ml. It was used at a speed of 26,000 r.p.m. (55,000 × g) for periods of 60 to 120 minutes.

In the experiments on the effects of hyaluronidase a Spinco Model L preparative ultracentrifuge was used. For small volumes of solutions a #40 rotor with a capacity of 144 ml. was run at 40,000 r.p.m. (107,000 × g) for periods of 30 to 60 minutes.

[4] In a few tests with 10-day embryos a 20-mesh gauze was used.
[5] Specialized Instruments Corporation, Belmont, California.
[6] By courtesy of Dr. George H. Hogeboom, Laboratory of Biology, National Cancer Institute.

For larger volumes of solution a #20 rotor with a capacity of 1,200 ml. was run at 20,000 r.p.m. (43,000 × g) for 60 to 90 minutes.

Filters and methods of handling

Selas unglazed porcelain filter candles [7] in various sizes and porosities were used in this investigation. This type of filter has been used routinely in this laboratory for several years for sterilizing horse serum, saline, and other media. An unlisted shape designated FPS, prepared especially for work in this laboratory, was used. The porcelain headpiece of this design has a nipple ranging in length from 2 inches on the smaller filters to 2½ inches on the larger filters (fig. 1). The nipple is of uniform diameter throughout its length so that rubber tubing may be slipped over it readily.

Preliminary clarification and polishing filtrations were carried out in different experiments with filter candles of porosity numbers XF, XFF, 10, and 01. The characteristics of these filters are shown in table 1. Porosity XFF, with a wall thickness of 3 to 4 mm., was the most satisfactory of these and was finally adopted as standard for preliminary clarification of the extract. Selas candles of #03 porosity were routinely used for all sterilizing filtrations.

Various sizes of filter candles were used in different experiments, the smaller sizes generally being used to filter smaller volumes of solution.

In early experiments considerable variability occurred in the results obtained from different sizes of Selas filter candles. Therefore several filter candles of three different makes were sawed open. It was found that, apparently in order to give mechanical strength proportional to the surface area, the wall of the large filters had been made thicker than that of the small filters of the same porosity. To eliminate this variable a special series of Selas filters of the #03 porosity was therefore obtained in which the wall thickness for all sizes was approximately uniform, 2.5 to 4.0 mm. Figure 1 shows eight different filter candles sawed open in order to display the range of variation in wall thickness between various sizes and three different makes. All of these candles were taken at random from the current laboratory supply.

The general design of the equipment used in these filtrations is shown in text-figure 1. A cylinder of five percent CO_2 in air furnished the pressure for forcing the embryo extract through the filter.[8] The pressure was regulated by means of a Hoke two-stage pressure-reducing valve [9] fitted with a gauge having a range of 30 p.s.i. and with the range 0 to 10 p.s.i. graduated to ounces. The coarser filters required pressures seldom greater than 2 p.s.i. for ready passage of the extract; for the #03 filter, pressure seldom exceeded 15 p.s.i. With a few lots of solution, when it was necessary to establish definitely that the #03 filter had clogged, pressure was raised to 18 p.s.i.

Porosity of each filter candle was established initially by a determination of the bubbling pressure, and was rechecked frequently. To determine bubbling pressure the filter candle was immersed in distilled water for several minutes. Water was then drawn through the candle by vacuum, displacing air from the filter wall. The water contained in the lumen of the candle was drained out, the filter was again immersed, and gas pressure from a cylinder of nitrogen or air was exerted on the inner surface of the filter.[10] This pressure was raised slowly until the internal gas pressure on the filter approached the anticipated bubbling pressure. Beyond this point the pressure was raised in one-pound increments, allowing about one minute for each increment before

[7] Selas Corporation of America, Erie Avenue and D Street, Philadelphia 34, Pa. Selas filters were introduced for general laboratory use after the comprehensive review of bacterial filters and filtration techniques by Morton (5) was published in 1938.

[8] For filtration of solutions that contain a bicarbonate buffer, a pressure system was essential, because vacuum caused a rapid loss of CO_2 from the bicarbonate buffer with resultant alkaline shift of *p*H to 8.4 or higher.

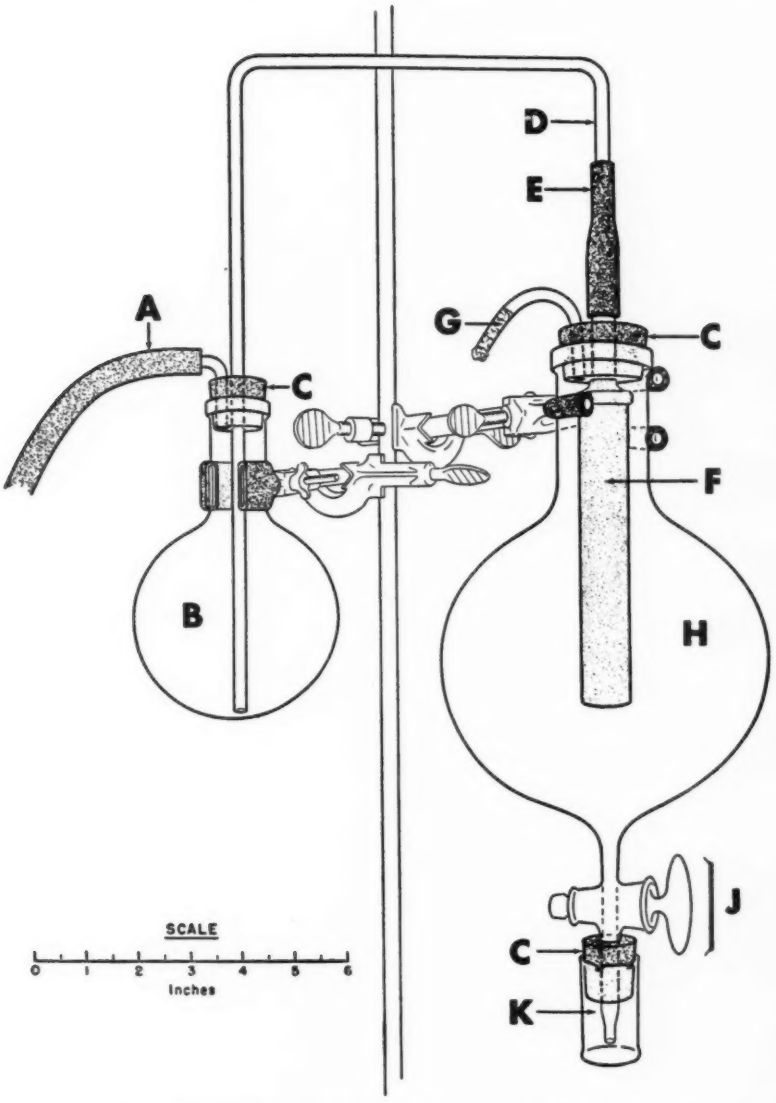
[9] Hoke Incorporated, Englewood, N. J.

[10] This cylinder was fitted with a two-stage Hoke reducer having a low-pressure gauge graduated in 2-pound divisions from 0 to 60 p.s.i.

TABLE 1.—*Physical characteristics of filters*

Brand and material	Porosity designation	Bubbling pressure		Pore diameter		Purpose listed
		Listed (*p. s. i.*)	Found* (*p. s. i.*)	Maximum (μ)	Mean (μ)	
Selas† porcelain	XF	0.45	ND‡	100	.....	Preliminary clarification.
	XFF	1.0	1	40	.....	Preliminary clarification.
	10	5.0	5	8.8	.....	Clarification.
	01	7.0	7–8	6.0	.....	Fine clarification.
	02	25	20–25	1.7	.....	Sterilization.
	03	35	27–33**	1.2	.....	Sterilization with high safety factor.
Mandler§ kieselguhr	Preliminary	2–5	ND	.....	(‖)	Clarification.
	Regular	6–9	ND	.....	.....	General sterilization.
	Fine	10–16	ND	.....	.....	Sterilization with high safety factor.
Berkefeld¶ kieselguhr	V(coarse)	(‖)	ND	.....	8–12	Clarification.
	N(normal)	.....	ND	.....	5–7	General sterilization.
	W(dense)	.....	ND	.....	3–4	Sterilization with high safety factor.

* Determined in this laboratory.
† Manufacturer's data from "Microporous Porcelain Filter Media," Selas Corporation of America, Scientific Equipment Division, Philadelphia 34, Pa., 1949.
‡ Not determined.
§ Allen Filter Company, Toledo, Ohio is the manufacturer. Data are cited from Morton (*5*) and Mudd (*4*).
‖ Morton (*5*) states that the three grades of Mandler and Berkefeld filters correspond closely. Therefore, it might be assumed that the mean pore diameters of the Mandler series are close to those given for the Berkefeld filters, and that the bubbling pressures of the Berkefeld series are close to those given for the Mandler filters.
¶ Manufacturer's data cited from Morton (*5*) and Mudd (*4*).
**The Selas #03 filter used in this experimental study fell within the bubbling pressure range of 27 to 33 p.s.i. At the present time, however, all Selas #03 filters being used conform with the specifications of the Selas Corporation in having bubbling pressures within the range 32 to 35 p.s.i.



TEXT-FIGURE 1.—Pressure filtration unit. *A*, heavy-walled rubber tubing from gas tank; *B*, 500 ml. pyrex round-bottom flask for pressure reservoir; *C*, rubber stoppers; *D*, heavy-walled pyrex tubing, 9 mm. O.D., 3 mm. bore; *E*, surgical latex rubber tubing bound tightly with heavy cord in order to prevent leaks; *F*, Selas filter candle, #03 porosity, 1×6-inch size; *G*, pyrex U-tube, packed with cotton, for escape of air from collecting flask; *H*, collecting flask modified from 1000-ml. round-bottom pyrex flask; *J*, delivery stopcock, 4–5 mm. bore; *K*, pyrex sleeve to protect delivery tip from contamination. Maximum safe gas pressure has been assumed to be about 15 p.s.i. Flask *B* is surrounded by a metal shield when the unit is in use.

again raising the pressure. The bubbling pressure [11] was established as that pressure at which observable small bubbles began to rise from any part of the filter surface. Through this study the range of bubbling pressure allowed for filters of the #03 porosity was 27 to 33 p.s.i. The bubbling pressures for all #03 filters used in any single experiment was held within a range of 2.5 p.s.i.

During the determination of the bubbling pressure, the distilled water in which the filter was immersed was contained in a lucite cylinder. The dimensions of this cylinder were approximately 6 inches internal diameter and ⅛ inch wall thickness; the lower end was formed by cementing a lucite sheet ¾ inch thick over the lower end of the cylinder. The transparent wall of this cylinder allowed the filter to be kept under continuous observation for bubbles and at the same time provided a safe shield in case a filter should explode under pressure.

After filtration each filter was reverse-washed by suction, first in a solution containing approximately 7.5 gm. NaCl and 2.5 gm. $NaHCO_3$ per liter, second in tap water, and finally in distilled water. If a bubbling pressure determination indicated any evidence of clogging or if the filter surface showed stain, *e.g.* iron oxide, the filter was digested in 50 percent HCl at 100° C. for 8 hours and then was reverse-washed with distilled water. The special acid digestion apparatus is shown in text-figure 2. The candle was dried and then fired in a thermostatically controlled muffle furnace at 1600° F. for 8 hours. The bubbling pressure was again determined in cases where a recheck seemed advisable. The filter was reverse-washed in triple-distilled water and was finally dried for storage.

Several modifications of this cleaning procedure have recently been made or are planned. 1) Only distilled water is used in washing these filters in order to avoid any heavy metal contamination. 2) Plans are being made to replace the glass digestion tube by a glass-lined metal tank of large size.

Filter candles were stored in a closed cabinet. The filter was held nearly vertical, with the porous element uppermost, by inserting the glazed porcelain connector in a hole in a supporting block. Each candle was protected further against dust and vapor adsorption by means of a pyrex tube which fitted snugly over the porous element.[12]

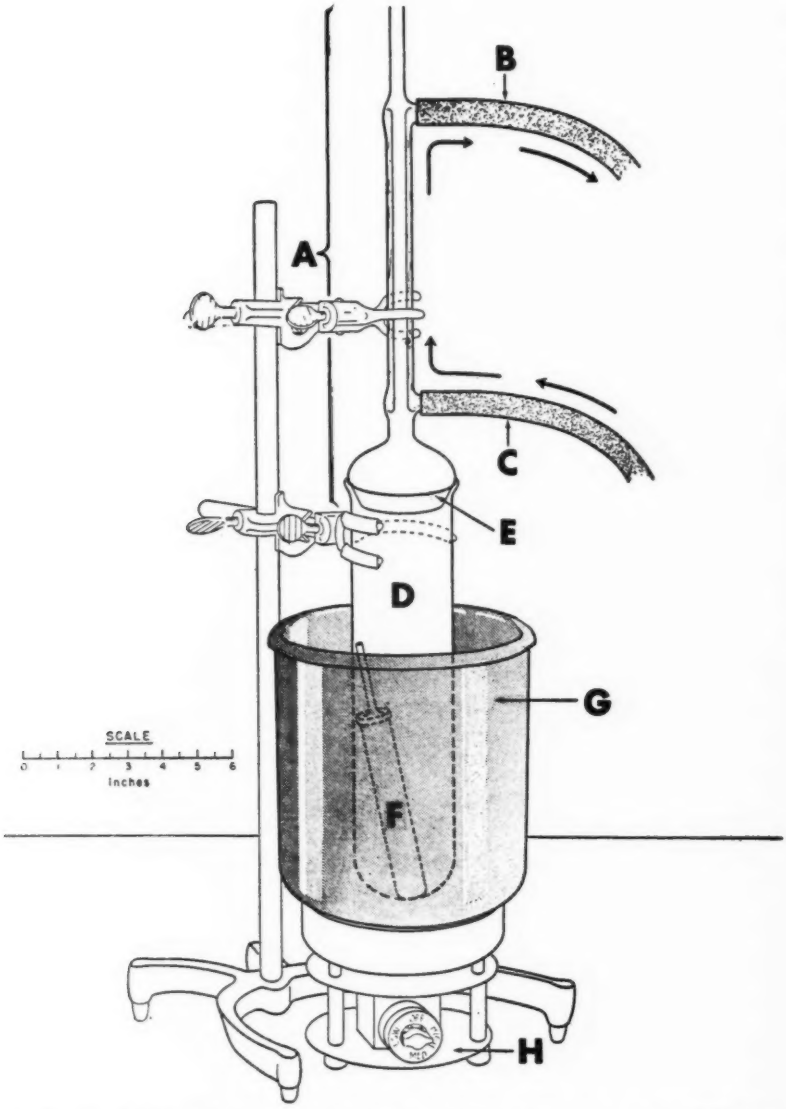
Hyaluronidase

The hyaluronidase in this investigation was a commercial product prepared from bovine testes.[13] Either the dry material was added directly to the embryo extract, or a solution of hyaluronidase in triple-distilled water in known concentration was added to the extract. The volume of hyaluronidase solution added to the extract was in all cases held so low that its diluting effect was negligible. The final concentrations used in various experiments ranged from 0.0001 to 5.0 mg. per 100 ml. of embryo extract. All hyaluronidase solutions used were either fresh or not more than eight days old. When more than one day was to elapse between preparation and use the solution was sterilized by filtration. The temperature of the embryo extract when hyaluronidase was added, either immediately before or just after ultracentrifugation, was usually 3° to 4° C. In the initial studies with the enzyme, the extract was warmed to about 30° to 35° C. for a least one hour before filtration in order to favor the maximum degree of enzyme action. This warming-up period was omitted in

[11] The bubbling pressure for #03 filters as established in this way was consistently about 2 to 8 pounds lower than that given by the manufacturer. A communication from Mr. George V. Jordan, Jr., of the Selas Corporation of America, states that this is probably due to the fact that in their tests a much shorter interval was allowed for equilibrium to be reached after each pressure change, and that in future tests the longer interval would be used.

[12] Before this method of storage was used the first fluid to pass through a sterile Selas filter of #03 porosity showed a marked yellowish discoloration. This color was so pronounced that the first few milliliters in the collecting funnel were drawn off separately and discarded. Mr. Jordan of the Selas Company states that this discoloration is caused by organic vapors adsorbed from the air by the porous filter.

[13] For some preliminary tests the hyaluronidase was supplied by Bios Laboratories, 17 West 60th Street, New York 23, N. Y. All of the hyaluronidase used in the later experiments and for routine laboratory preparation of filtered extract was supplied by the Armour Laboratories, Chemical Research and Development Department, Chicago 9, Ill. Specifications for the Armour product were: moisture 12 percent, potency 200 Turbidity Reducing Units per mg. (*5*).



TEXT-FIGURE 2.—Acid digestion and reflux condenser assembly for cleaning Selas filters. *A*, reflux condenser; *B*, rubber tubing for water outlet; *C*, rubber tubing for water inlet; *D*, heavy-walled pyrex tube (3½×15 inches) for acid; *E*, ground-glass contact surfaces; *F*, Selas filter (1×6-inch size); *G*, stainless steel water bath (9×9-inch size); *H*, hot plate.

later work, particularly when hyaluronidase-treated extract was prepared in large amounts for routine use, since it did not prove to be essential for optimum filtrability of the extract provided hyaluronidase was added to the extract before ultracentrifugation.

Determination of viscosity

Ostwald-type viscosity pipettes[14] were used to determine changes in viscosity of embryo extract. Pipettes were tested with distilled water, Earle's balanced salt solution, and embryo extract. The pipettes used in this study had outflow times of 48 to 75 seconds for 6 ml. of distilled water, and up to 130 seconds for the more viscous lots of embryo extract. At least three consecutive determinations of outflow time were made on each sample. Viscosity determinations were made at a water-bath temperature of 30° C.

Changes in viscosity were expressed in terms of relative viscosity $=\frac{t_1-t_2}{t_2}$ where t_1 is the outflow time of the test solution and t_2 is the outflow time of distilled water or of Earle's saline. This saline and distilled water were found to have essentially the same outflow time (within ± 1 second).

Determination of nitrogen and phosphorus

Determinations were made in the Microanalytical Laboratory of the National Institutes of Health under the supervision of Dr. William C. Alford. Total nitrogen was determined by the modified micro-Kjeldahl method of Ma and Zuazaga (*7*). Nonprotein nitrogen was determined by the direct nesslerization micro-Kjeldahl method of Koch and McMeekin (*8*). Protein nitrogen was calculated by taking the difference between the total nitrogen and nonprotein nitrogen analyses. Total phosphorus was determined by the colorimetric micro method for plant materials (*9*), modified to include rapid wet ashing.

Criteria of satisfactory filtrability as determined in earlier studies

In the earlier studies the satisfactory filtrability of any solution was evaluated from the following criteria: 1) the passage of a satisfactory volume of solution through the filter, and demonstration that undue prolongation of the filtration time and unduly rapid clogging of the filter did not occur; 2) the demonstration by chemical analysis that there had been no substantial decrease in either nitrogen or phosphorus concentration of the solution during filtration.

In later studies these criteria were supplemented by actual study of living cultures in media of which the solution was a component.

Tissue culture methods

Short-term quantitative experiments with strain L cells.—Strain L cells of single-cell origin (*10*) were used for preliminary testing of the activity of the embryo extract in promoting cell proliferation. In each experiment a cell suspension was prepared (*11*) in horse serum. The nuclei were enumerated in aliquots of this suspension (*12*). Experimental cultures were planted on cellophane substrates in T-12 flasks.[15] Measured volumes (1 to 5 ml.) of the extract to be tested and Earle's balanced saline were placed in each culture flask. One milliliter of the cell suspension was then added, the flask was gassed with 5 percent CO_2 in air, and sealed with a rubber stopper. Three experiments, designated as series 1254, 1291, and 1305 respectively, are reported. In the first two, the relative percentages of embryo extract, horse serum, and saline were 20, 40, and 40; in experiment 1305 the percentages used were 25, 35, and 40 respectively.

Thrice weekly a measured amount of the fluid in each culture was replaced. At the first renewal, a 2-ml. aliquot of fluid was withdrawn from each culture and replaced

[14] The pipettes were fabricated for this work by Mr. Frederick Highhouse, Head, Instrumentation Section, National Cancer Institute.

[15] The detailed design of this flask will be given at a later date.

by only 1 ml. of fresh fluid; at each subsequent fluid change the volume of fluid withdrawn and renewed was the same, 1 ml.

The three experiments were continued for 10, 17, and 13 days respectively. Periodically during the course of each experiment, groups of 4 cultures were removed from each set for enumeration of their nuclei (*12*). Representative cultures of each set were photographed at 200 × in order to record changes in cell morphology which might occur as a result of using filtered embryo extract in the media. The photomicrographic procedures used have been described previously (*13, 14*).

Longer-term tissue culture experiments with four cell strains.—Four cell strains were used to test the cumulative effect of filtered embryo extract on tissue cultures over several generations during a period of 149 days. These were strain L mouse fibroblasts of single-cell origin (#929) (*10*), another strain of mouse fibroblasts (#1293) (*15*), a strain of mouse liver cells (#721) (*16*), and a strain of chicken fibroblasts (#1336). Cultures were carried on cellophane substrates in D-3.5 Carrel flasks. Each strain was carried in eight flasks, of which four received filtered embryo extract and four received untreated extract in the media. Culture flasks were gassed with 5 percent CO_2 in air at each fluid change. Periodic transfers were made to new culture flasks as crowding of the cultures indicated.

Longer-term tissue culture experiments with many cell strains and large lots of embryo extract.—A final test of the long-term cumulative effect of filtered chick-embryo extract on tissue cultures was begun October 19, 1951, when approximately half of the tissue cultures of each of the various cell strains being carried in this laboratory were placed in media containing filtered chick-embryo extract. Cultures in the other half were maintained as before with standard media containing untreated embryo extract. The two groups of tissue cultures were carried under identical conditions in all other respects. This large-scale laboratory test was continued for five months. Cultures were grown in various types and sizes of flasks and on various substrates including cellophane, glass, and plasma clot, by methods previously described.

Experiments and Results

Physico-chemical Changes of Embryo Extract by Ultracentrifugation or Hyaluronidase or Their Combination

Preliminary filtration experiments with stored embryo extract

Filtrability with low-speed centrifugation.—Experiments 1 and 2 were carried out to confirm earlier observations in this laboratory that chick-embryo extract would not pass through a #03 Selas filter following centrifugation at 1,600 × g for 60 minutes and preliminary clarification through a Selas XF filter. The extract passed through the clarifying filter readily. Results presented in table 2 show that the #03 filters clogged when only limited volumes of the clarified extract had passed. Dilution of the extract to the 1:3 level in experiment 1 improved filtration only slightly. Two preliminary clarifying filtrations in experiment 2 apparently failed to improve filtrability. Nitrogen was reduced somewhat by the final filtration, while phosphorus remained at about the same level.

Filtrability after ultracentrifugation.—Table 3 presents results of three experiments (3, 4, and 5) to determine whether ultracentrifugation of stored embryo extract would promote its filtrability. These experiments together with results from numerous other tests indicated that while perhaps an occasional lot of stored embryo extract could be filtered satisfactorily following preliminary ultracentrifugation and clarification, as in experiment 4, most lots of stored embryo extract rapidly clogged the #03 filter.

Clogging of the filters in experiments 3 and 5 was accompanied by a sharp decrease in nitrogen concentration during filtration. However, complete filtration in experiment 4 involved no appreciable retention of nitrogen by the filter since the nitrogen in the filtrate was nearly the same as it had been before filtration.

TABLE 2.—*Filtrability of stored chick-embryo extract following preliminary centrifugation at 1,600 × g for 60 minutes and clarifying filtration*

Experiment number:	1		2
Lot numbers:	1240–1252		1401–1443
Age of stored extract (*months*):	12		1–4
Filtration:			
Dilution of extract to be filtered	1:1	1:3	1:1
Amount of clarified extract (*ml.*)	305*	615*	100†
Size of #03 filter candle (*in.*)	1×6	1×6	⅝×6
Maximum pressure used (*p.s.i.*)	12	12	12
Duration of filtration period (*min.*)	50	50	30
Final filtrate obtained (*ml.*)‡	160	250	40
Filter behavior	clogged	clogged	clogged
Chemical composition (mg./100 ml.):			
Total nitrogen in clarified extract	117	55	118
" " " #03 filtrate	97	55	94
Total phosphorus in clarified extract	24.5	13.5	22.4
" " " #03 filtrate	23.5	13.0	23.7§

*Clarified once through a #XF filter.

†Clarified twice, through a #XFF filter and through a #10 filter.

‡In any filtration a certain amount of fluid was retained in the porous wall of the filter. For the four filter candle sizes used at various times in this investigation, namely ⅝ × 2, ⅝ × 6, 1 × 6, and 1⅛ × 8 inches, the volumes o fluid so retained were approximately 2, 6, 13, and 30 ml. respectively.

§See table 5, footnote †.

The results of experiment 3 as outlined in table 3 made it seem possible that ultracentrifugation for 30 minutes had been inadequate to allow filtration. Subsequent additional centrifugation for 120 minutes, not recorded in table 3, did not appreciably improve filtrability.

Preliminary filtration experiments with freshly prepared embryo extract

The preceding experiments were done with embryo extract that had been stored at −10° C. for 6 to 12 months. It seemed possible that freshly prepared embryo extract might have different filtrability characteristics with respect to ultracentrifugation and other factors.

Filtrability after ultracentrifugation.—Table 4 presents data from two experiments to test this point. Severe clogging occurred in both experiments. In experiment 6 the filters retained considerable nitrogen and phosphorus; nitrogen and phosphorus analyses were not made in experiment 7. These results were confirmed by other filtration experiments in which freshly prepared embryo extract was ultracentrifuged.

Effect of separation of the fatty components of ultracentrifuged extract on filtrability.—The possibility was considered that the fatty substances in the embryo extract might be clogging the filters. In order to test this possibility a crude separation of the ultracentrifugate into a fatty part and a clear part was made in experiments 8 and 9 (table 5) by pipetting off the upper turbid layer. When these two parts were filtered separately in experiment 8, the fatty part passed the Selas #03 filter readily at both 1:1 and 1:3 dilutions; the clear extract clogged the filter. In experiment 9 the fatty fraction was combined with part of the clear extract after sludging filtration (and was designated as "clear-fatty recombination") in order to have more nearly equal volumes in the two fractions to be filtered. Clogging stopped filtration at about the same stage in both

cases. Nitrogen and phosphorus in the two fractions declined at similar rates during ultracentrifugation and clarifying filtration.

TABLE 3.—*Effect of ultracentrifugation on filtrability of stored chick-embryo extract following preliminary centrifugation at 1,600 × g for 30 minutes*

Experiment number:	3	4		5
Lot numbers:	1228–1233	1234–1239		
Age of stored extract (*months*):	12	12		6
Ultracentrifugation:				
Speed (*r.p.m.*)	26000	26000		20500
Relative centrifugal force (*g*)	55000	55000		46000
Duration of maximum centrifugation speed (*min.*)	30	60		30
Dilution of extract to be filtered	1:1	1:1	1:3	1:1
Filtration:				
Preliminary clarifying filter porosity	None	XF	XF	XFF
Amount of clarified extract (*ml.*)	200	215	415	90
#03 filter, size (*in.*)	1×6	1×6	1×6	⅝×6
Bubbling pressure of #03 filter (*p.s.i.*)	ND*	ND	ND	28.5
Maximum pressure used (*p.s.i.*)	15	10	10	14
Duration of filtration period (*min.*)	10	20	30	15
Final filtrate obtained (*ml.*)†	15	200	400	47
Filter behavior	Clogged	No clogging	No clogging	Clogged
Chemical composition (mg./100 ml.):				
Total nitrogen in ultracentrifugate	112	106	52	222‡
Total nitrogen in #03 filtrate	78	103	54	188
Total phosphorus in ultracentrifugate	24.6	22.6	11.4	ND*
Total phosphorus in #03 filtrate	22.0	22.1	11.4	ND

*In this table and in subsequent tables ND indicates cases where embryo extract was available at various stages in the experiment but no determination was made.

†See table 2, footnote ‡.

‡Before ultracentrifugation.

The data indicated that the fatty layers of the supernatant from ultracentrifugation caused no clogging of the filter; in fact it appeared that these components even slightly facilitated filtration of the extract.

Comparison of filtrability through Berkefeld "N" and Selas #03 filters.—In experiment 10 the 1:1 extract from 190 nine-day and 30 ten-day embryos was pooled and then divided into lot A, to be ultracentrifuged, and control lot B, not ultracentrifuged. The plan for the ensuing experimental procedure is shown in text-figure 3, and the quantitative data are presented in table 6. Clarified extract A gradually clogged the Selas #03 filter. Extract B gradually clogged the #01 clarifying filter used. It was obviously impractical to try to pass it through the finer #03 filter, so no attempt was made to do so.

TABLE 4.—*Effect of ultracentrifugation on filtrability of freshly prepared chick-embryo extract following preliminary centrifugation at 1,600 × g for 15 minutes*

Experiment number:	6	7
Ultracentrifugation:		
Speed (*r.p.m.*)	26000	26000
Relative centrifugal force (*g*)	55000	55000
Duration of maximum centrifugation speed (*min.*)	60	60
Dilution of extract to be filtered	1:1	1:1
Filtration:		
Preliminary clarifying filter porosity	XFF	XF
Amount of clarified extract (*ml.*)	63	250
#03 filter, size (*in.*)	⅝×6	1×6
Bubbling pressure of #03 filter (*p.s.i.*)	30	ND
Maximum pressure used (*p.s.i.*)	15	15
Duration of filtration period (*min.*)	30	30
Final filtrate obtained (*ml.*)*	20	10
Filter behavior	Clogged	Clogged
Chemical composition (mg. /100 ml.):		
Total nitrogen in ultracentrifugate	55	ND
" " " #03 filtrate	35	ND
Total phosphorus in ultracentrifugate	21.1	ND
" " " #03 filtrate	18.1	ND

*See table 2, footnote ‡.

At this point, "N" Berkefeld filters were introduced into the procedure. Both lots passed through Berkefeld "N" filters readily; the resultant filtrates then both passed easily through Selas #03 filters. Losses of nitrogen and phosphorus from both lots during the Berkefeld filtration were serious, however—certainly from lot A and probably from lot B. Consequently no further tests were made with diatomaceous earth filters.

The action of hyaluronidase in reducing viscosity of chick-embryo extract

The lack of any adequate improvement in the filtrability of chick-embryo extract as a result of ultracentrifugation led to a consideration of the possibility that viscosity of the extract was associated with clogging of the filters. It seemed possible that the relatively high viscosity of chick-embryo extract might be due to hyaluronic acid from the embryonic tissue; if so, hyaluronidase should reduce this viscosity and might enhance the filtrability of the extract.

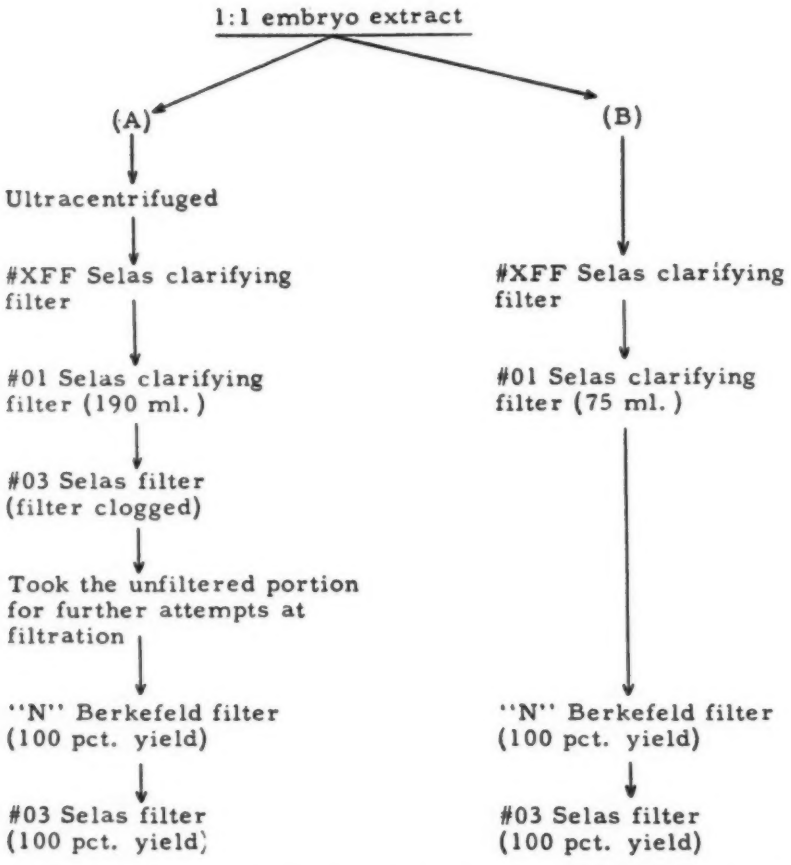
The effect of hyaluronidase upon the final viscosity of embryo extract after treatment for periods of from 5 minutes to several hours was therefore tested under various conditions in a series of experiments. Hyaluronidase was first used in a final concentration of 5 mg. per 100 ml. and within 5 minutes reduced the viscosity of freshly made 1:1 extract of 9-day chick embryos from 0.57 to 0.24 relative viscosity. This test showed clearly that the substrate for hyaluronidase, presumably hyaluronic acid, was the agent responsible for the high viscosity of chick-embryo extract, and that hyaluronidase could lower this viscosity.

TABLE 5.—*The effect of separation of the fatty layer from the clear parts of ultracentrifuged fresh chick-embryo extract on its filtrability and composition*

Experiment number:	8				9	
Ultracentrifugation:						
Speed (*r.p.m.*)	26000		26000		26000	
Relative centrifugal force (*g*)	55000		55000		55000	
Duration of maximum centrifugation speed (*min.*)	120		120		90	
Dilution of extract to be filtered	1:1		1:3		1:1	
Layer of ultracentrifugate separated	Clear	Fatty	Clear	Fatty	Clear	Fatty
Filtration:						
First clarifying filter porosity number	10	10	10	10	XFF	XFF
Second clarifying filter porosity number					10	10
Amount of clarified extract (*ml.*)	155	20	95	20	290	85
Recombined fractions					Clear	Cl. Fat
Amounts of recombined fractions (*ml.*)					110	150
#03 filter, size (*in.*)	1×6	⅝×6	⅝×6	⅝×6	⅝×6	⅝×6
Bubbling pressure of fine filter (*p.s.i.*)	ND	31	25	30	28½	28½
Maximum pressure used (*p.s.i.*)	12	10	12	10	18	18
Duration of filtration period (*min.*)	17	10	17	5	45	45
Final filtrate obtained (*ml.*)*	40	15	55	15	60	75
Filter behavior	Clogged	No clogging	Clogged	No clogging	Clogged	Clogged
Chemical composition (mg./100 ml.):						
Total Nitrogen:						
In first ultracentrifugate					166	166
In second ultracentrifugate						113
In first clarified filtrate					126	104
In second clarified filtrate					118†	104†
In #03 filtrate					120†	110†
Total Phosphorus:						
In first ultracentrifugate					29.1	29.1
In second ultracentrifugate						21.6
In first clarified filtrate					23.1	21.1
In second clarified filtrate					20.6†	20.1†
In #03 filtrate					24.6†	24.6†

*See table 2, footnote ‡

†In this table and in subsequent tables there is frequently a slight increase in concentrations of nitrogen and phosphorus during #03 filtration. This increase appears to be the result of preferential absorption of water in the filter candle.


1:1 embryo extract
(A)
(B)
Ultracentrifuged
#XFF Selas clarifying filter
#01 Selas clarifying filter (190 ml.)
#03 Selas filter (filter clogged)
Took the unfiltered portion for further attempts at filtration
"N" Berkefeld filter (100 pct. yield)
#03 Selas filter (100 pct. yield)
#XFF Selas clarifying filter
#01 Selas clarifying filter (75 ml.)
"N" Berkefeld filter (100 pct. yield)
#03 Selas filter (100 pct. yield)

TEXT-FIGURE 3.—Plan of experiment 10: the effect of ultracentrifugation and kind of filter on filtrability of fresh chick-embryo extract.

The effect of concentrations of hyaluronidase lower than 5 mg. per 100 ml. were studied in another test (table 7). The reduction in viscosity of embryo extract was directly correlated with the increasing concentration of hyaluronidase over the range studied.

The rapidity of the viscosity-reducing action of hyaluronidase at 30° C. and at intermediate concentrations is shown in table 8. It is obvious that hyaluronidase at concentrations from 0.5 to 5.0 mg. per 100 ml. exerted almost all of its viscosity reduction within the first 10 minutes. Little consistent decrease in viscosity occurred during the remaining 156 minutes of the test. In another experiment lower concentrations of hyaluronidase, 0.014, 0.072, and 0.360 mg. per 100 ml., did not bring about their maximum effect on viscosity until two or three hours had elapsed. Consequently in later studies at least one hour was allowed between the time of addition

of hyaluronidase and the time of filtration in order to insure the maximum degree of viscosity reduction.

TABLE 6.—*Experiment 10: Effect of ultracentrifugation and kind of filter on the filtrability and composition of fresh chick-embryo extract.*

(For sequence of procedure see text-figure 3)

	Experiment No. 10			
Age of embryos (*days*)	9 and 10			
Dilution of pulp with saline	1:1			
Ultracentrifugation:	A		B	
Speed (*r.p.m.*)	26000		(*)	
Relative centrifugal force (*g*)	57000		.....	
Duration of maximum centrifugation speed (*min.*)	90		.....	
Filtration:				
First clarifying filter porosity	XFF		XFF	
Second clarifying filter porosity	01		01	
Amount of clarified extract (*ml.*)	190		75	
#03 Selas filter, size (*in.*)	⅝×6		(†)	
Bubbling pressure of #03 filter (*p.s.i.*)	28		.....	
Maximum pressure used (*p.s.i.*)	18		.....	
Duration of filtration period (*min.*)	60		.....	
Final filtrate obtained (*ml.*)‡	80		.....	
Filter behavior	Clogged		.....	
"N" Berkefeld filter, size (*in.*)	1×6		1×6	
Maximum pressure used (*p.s.i.*)	5		5	
Duration of filtration period (*min.*)	5		5	
Filtrate obtained (*ml.*)	75		70	
Percentage passed	100		100	
#03 Selas filter, size (*in.*)	⅝×6		⅝×6	
Bubbling pressure of #03 filter (*p.s.i.*)	29		29½	
Duration of filtration period (*min.*)	5		5	
Percentage passed	100		100	
Chemical composition (mg./100 ml.):	N	P	N	P
Before ultracentrifugation	143	23.6	.....	.....
In supernatant from ultracentrifugation	141	22.2	148	22.7
In the first clarified filtrate	135	22.8	141	22.2
In the second clarified filtrate	132	23.0	130	23.0
In the first #03 filtrate	130	23.4	.....	.....
In the Berkefeld filtrate	47	10.7	ND	ND
In the final #03 filtrate	36	10.7	43	10.3

*Ultracentrifugation omitted.
†The first Selas #03 filtration was omitted.
‡See table 2, footnote ‡.

The action of hyaluronidase in promoting filtrability of chick-embryo extract

The effect of hyaluronidase treatment on the filtrability of chick-embryo extract was studied under various conditions in a series of three experiments. The basic data of these experiments, numbers 12, 13, and 14, are outlined in table 9.

TABLE 7.—*Effect of ultracentrifugation and hyaluronidase on specific viscosity of chick-embryo extract**

	Before ultracentrifugation	After ultracentrifugation†					
Hyaluronidase (mg. per 100 ml. extract)	0	0	.0001	.0005	.0025	.0125	.0625
Relative viscosity	.54	.45	.41	.35	.30	.23	.22

*Extraction at 1:1 dilution from 9-day embryos; extract was stored at −10° C. one month before use. Viscosity was determined 3 to 3½ hours after addition of hyaluronidase.

†In #40 head at 40,000 r.p.m. (107,000 × g) for 30 minutes.

TABLE 8.—*Specific viscosity of chick-embryo extract in relation to concentration of hyaluronidase and time after addition of hyaluronidase**

Time after addition of enzyme (*minutes*)	Hyaluronidase added (*specific viscosity*)		Hyaluronidase added after ultracentrifugation (*specific viscosity*)		
0	.36	.36	.36	.36	.36
9–13	.36	.12	.18	.17	.08
14–18	ND	.14	.20	ND	.09
19–25	.35	.14	.18	.16	.09
26–30	.36	.14	.17	.16	ND
31–35	.36	.14	.16	.16	.07
36–41	.35	.13	ND	.16	ND
77–82	ND	.13	.15	.15	ND
162–166	ND	.12	.15	.14	.06
Concentration of hyaluronidase in mg. per 100 ml	0	0.5	0.5	1.0	5.0

*Extraction at 1:1 dilution from 9-day embryos. The extract was stored at −10° C. for nine days before use. It was thawed and then ultracentrifuged in #40 head at 40,000 r.p.m. (107,000 × g) for 30 minutes.

Effect of hyaluronidase on filtrability of ultracentrifuged chick-embryo extract.—Hyaluronidase greatly enhanced the filtrability of embryo extract in the experiments in which the extract was ultracentrifuged. Complete passage of treated embryo extract was attained in lot B_1 in experiment 14. While filtration was incomplete in two lots in experiment 13 and in lot A in experiment 14, the amounts of extract that did pass the filters satisfactorily in these experiments were comparatively large. Later tests indicated that the limiting factor responsible for eventual clogging in experiment 13 was an inadequate concentration of hyaluronidase (0.04 mg. per 100 ml.); the limiting factor in experiment 14 was probably either the omission of preliminary clarifying filtration or the comparative shortness of the ultracentrifugation time in relation to the large volume of extract to be filtered through one candle. The effectiveness of hyaluronidase in improving filtrability is emphasized by the fact that the corresponding control lots of untreated extract clogged the #03 filter when only much smaller volumes of filtrate had been obtained.

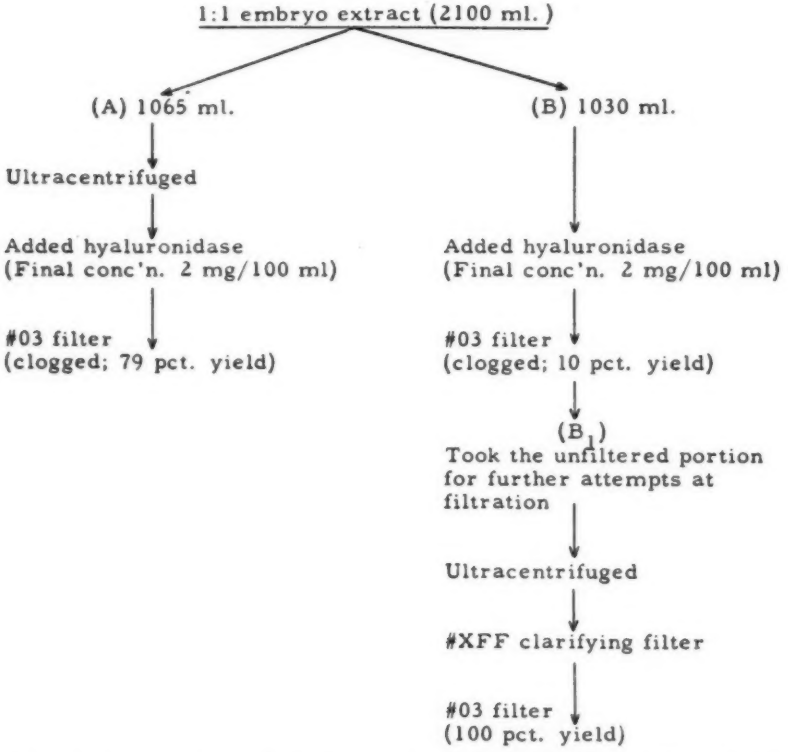
Effect of preliminary clarification on filtrability.—In early studies preliminary clarification through a coarse filter was assumed to be advisable.

TABLE 9.—*Effect of hyaluronidase and ultracentrifugation on filtrability, composition, and viscosity of 1:1 chick-embryo extract. (For sequence of procedures in experiment 14 see text-figure 4)*

	Experiment No. 12				Experiment No. 13			Experiment No. 14		
Age of embryos (*days*)	9		10		9			9		
Age of stored extract (*months*)	1 to 4		(Fresh)		1 to 2¼			¼ to 3½		
Lot numbers	1412 to 1438							1559 to 1601		
Ultracentrifugation:								A	B	B_1
Speed (*r.p.m.*)	(*)		(*)		20000			20000	None	20000
Relative centrifugal force (*g*)					43000			43000		43000
Duration of maximum centrifugation speed (*min.*)					30			40		45
Hyaluronidase:										
Final concentration in extract (*mg./100 ml.*)	None	2	None	2	None	0.4	0.04	2	2	2
When added (before or after ultracentrifugation)						Before	Before	After		Before
Filtration:										
First clarifying filter porosity	XFF	XFF	XFF	XFF	XFF	XFF	XFF			XFF
Second clarifying filter porosity	.10	.10	.10	.10						
Amount of clarified extract (*ml.*)	100	700	50	350	175	320	400	1065	1030	730
Fine (#03) filter, size (*in.*)	5/16×6	1×6	5/16×6	1×6	1×6	1×6	1×6	1½×8	1½×8	1½×8
Bubbling pressure of #03 filter (*p.s.i.*)	29	28	36	30	28	30	29½	30	29½	31½
Maximum pressure used (*p.s.i.*)	12	15	15	15	12	12	12	7½	?	6
Duration of filtration period (*min.*)	30	65	65	60	40	55	85	100	20	18
Final filtrate obtained (*ml.*)†	40	200	10	30	20	307	350	820	100	700
Filter behavior	Clogged	Clogged	Clogged	Clogged	Clogged	No clogging	Clogged	Clogged	Clogged	No clogging
Chemical composition (mg./100 ml.):										
Total nitrogen before ultracentrifugation	118	118	173	173	ND	144	150	115	115	115
Total nitrogen in ultracentrifugate					142	132	139	108		108
Total nitrogen after hyaluronidase was added		122		164				105	115	
Total nitrogen in clarified extract	ND	ND	ND	ND	ND	ND	ND			ND
Total nitrogen in final filtrate	94	116	134	138	110	132	136	102	89	105
Total phosphorus before ultracentrifugation	23.3	23.3	30.1	30.1	ND	27.1	27.0	ND	ND	ND
Total phosphorus in ultracentrifugate					25.8	24.6	25.6	ND		ND
Total phosphorus after hyaluronidase was added		22.4		31.5				ND	ND	
Total phosphorus in clarified extract	ND	ND	ND	ND	ND	ND	ND			ND
Total phosphorus in final filtrate	23.7	23.7	40.1	29.7	24.6	25.0	25.2	ND	ND	ND
Specific viscosity before ultracentrifugation:	.35	.35	.75	.75	.45	.14	.21	.30	.30	.10
Specific viscosity of ultracentrifugate					.40	.13	.17	.24		.12
Specific viscosity after hyaluronidase was added		.32		.23				.12	.10	
Specific viscosity of clarified extract	ND	ND	ND	ND	ND	ND	ND			ND
Specific viscosity of final filtrate	.18	.07	.13	.20	.18	.11	.13	.11	.08	.10

*Centrifuged only in low-speed centrifuge at 2,300 r.p.m. (1,600 × g) for 60 minutes. Stored extract from 9-day embryos was centrifuged again after thawing; fresh extract from 10-day embryos was centrifuged after extraction, and was not frozen. †See table 2, footnote ‡.

A test of this assumption, with hyaluronidase-treated extract, was made in experiment 14 (text-figure 4 and table 9). Preliminary clarification filtration was omitted from the procedure for both lot A and lot B, but was included for lot B_1. Comparison of the filtrability of lot A and of B_1 indicates that preliminary passage of lot B_1 through a clarifying filter did slightly increase its filtrability. Other experiments not detailed here showed that a preliminary clarification filtration was necessary only when the viscosity of a given lot of extract seemed to be unusually high.



TEXT-FIGURE 4.—Plan of experiment 14: the effect of ultracentrifugation and clarifying filtration on the filtrability of stored chick-embryo extract treated with hyaluronidase.

Effect of hyaluronidase alone on filtrability.—The possibility that hyaluronidase alone, without ultracentrifugation, could bring about satisfactory filtrability of embryo extract was considered in experiments 12 and 14. In experiment 12 hyaluronidase alone at a final concentration of 2 mg. per 100 ml. did not wholly prevent clogging of the #03 filters; it did retard clogging until the volume of filtrate was three or five times greater than that from the corresponding control extract. In experiment 14B a 1030-ml. lot of hyaluronidase-treated extract that had not been ultracentrifuged

clogged a large filter candle after only 10 percent of the extract had passed. From these results it seemed clear that the action of hyaluronidase alone was not adequate for satisfactory routine filtrability.

Effect of ultracentrifugation plus hyaluronidase on filtrability.—A definitive test of the effect of ultracentrifugation on hyaluronidase-treated extract was made in experiment 14. Ultracentrifuged lot A passed easily through the #03 filter to yield 820 ml. before clogging occurred. Lot B, which had not been ultracentrifuged, filtered slowly and finally clogged the filter when only 100 ml. of filtrate had passed. Lot B extract that did not pass the #03 filter was ultracentrifuged and clarified (as lot B_1 in text-figure 4 and in table 9). The extract then passed through the #03 filter rapidly and completely to yield 700 ml. of filtrate. The radical improvement in filtrability of lot B_1 as compared with that of original lot B was clearly due to ultracentrifugation. From these results it appears that ultracentrifugation is necessary in addition to hyaluronidase treatment in order to obtain an extract satisfactorily filtrable. Although the low viscosities of the extracts in lots A, B, and B_1 were practically identical, these three lots were quite different in their filtrability. It seemed apparent that ultracentrifugation had removed some filter-clogging particulate components from the extracts in lots A and B_1.

Effect of sequence of hyaluronidase treatment and ultracentrifugation on filtrability.—In a number of experiments not detailed here, as well as those given in text-figure 4 and table 9, it was found that hyaluronidase added before ultracentrifugation was fully as effective as when added afterward.

The Effect of Ultracentrifuged and Hyaluronidase-Treated Chick-Embryo Extract on Proliferation of Cells in Tissue Culture

Short-term quantitative experiments with strain L cells

The effect of hyaluronidase-treated, ultracentrifuged, filtered chick-embryo extract on tissue cultures of strain L cells was first studied in three short-term experiments, numbers 1254, 1291, and 1305, in which proliferation was determined quantitatively. The preparation of these lots of embryo extract is outlined in table 10. The growth data for the three series are presented in text-figures 5, 6, and 7 as curves in which the averages of the determinations of numbers of cell nuclei per flask are plotted on a semilogarithmic grid against time of exposure in days.

An analysis of variance of the average nuclei counts of series 1254 and 1305 showed that the difference between the curves for untreated embryo extract and for extract treated with various concentrations of hyaluronidase in each experiment are either barely significant (series 1254) or not quite significant (series 1305). Crowding and consequent necrosis in the untreated cultures of series 1291 after 10 days made an analysis of variance untenable for that series. In all three experiments, however, the untreated control cultures did show slightly more rapid proliferation than did any of the treated cultures. Microscopic examination of the living

TABLE 10.—*Conditions of treatment and total nitrogen composition of fresh 1:1 chick-embryo extract used for strain L mouse cells in tissue culture**

	Series 1254			Series 1291								Series 1305					
Hyaluronidase, final concentration (*mg./100 ml.*)†	None	0.1	1.0	None		0.1		1.0		5.0		None		1.0		2.0	
Period for which extract was used ‡	Entire	Entire	Entire	First	Second	First	Second	First	Second	First	Second	First	Second	First	Second	First	Second
Lot number of embryo extract	**1493**	**1493**	**1493**	**1566**	**1570**	**1566**	**1570**	**1566**	**1570**	**1566**	**1570**	**1593–4**	**1596**	**1593–4**	**1596**	**1593–4**	**1596**
Clarifying filter, porosity No.		XFF	XFF			None	None	None	None	None	None	None	None	None	None	None	None
Amount of clarified extract or ultracentrifugate (*ml.*)		56	52			45	30	45	30	45	30			42	30	42	28
Fine (#03) filter, size (*in.*)		⅛×2	⅛×2			⅛×6	⅛×2	⅛×6	⅛×2	⅛×6	⅛×2			⅛×2	⅛×6	⅛×2	⅛×2
Bubbling pressure of #03 filter (*p.s.i.*)		28	29			28	?	29½	?	30	?			29	29	29½	30
Maximum pressure for filtration (*p.s.i.*)		11	8			5	5	5	5	5	5			7	6	7	6
Duration of filtration period (*min.*)		10	10			6	5	6	5	6	5			3	5	7	5
Final filtrate obtained (*ml.*)§		21	21			39	28	39	28	39	28			40	24	40	26
Filter behavior		Clogged ‖	Clogged			No clogging		No clogging		No clogging				No clogging		No clogging	
Total nitrogen in untreated extract (*mg./100 ml.*)	167.5			150	ND							ND	132				
Total nitrogen in ultracentrifugate		139.4	140.1			ND	ND	ND	ND	ND	ND			ND	ND	ND	ND
" " " in final filtrate		134.5	127.2			116	121	108	113	99	118			98	62	75	102

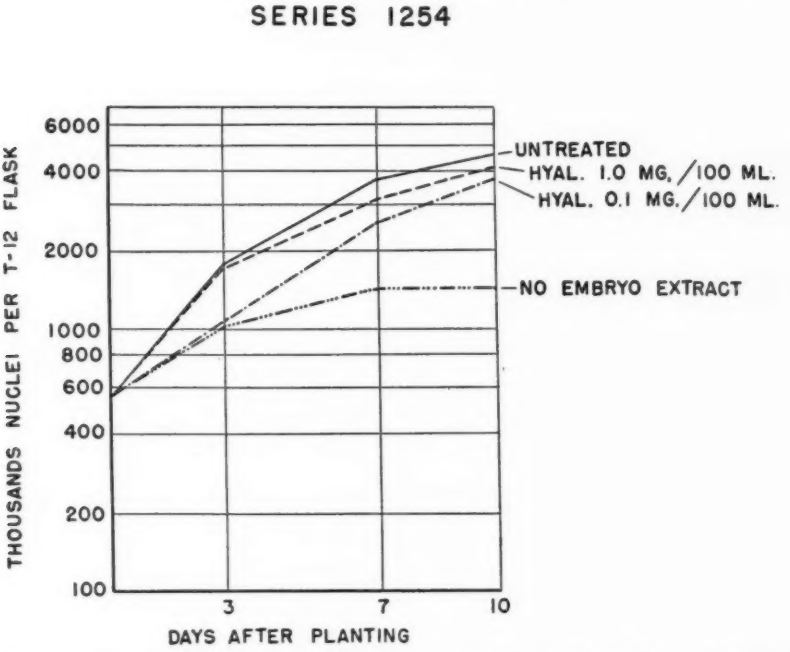
* Ultracentrifugation of all lots of treated extract used for these series was for 30 minutes at 40,000 r.p.m. (107,000 × g).

† Hyaluronidase was added to supernatant immediately after ultracentrifugation.

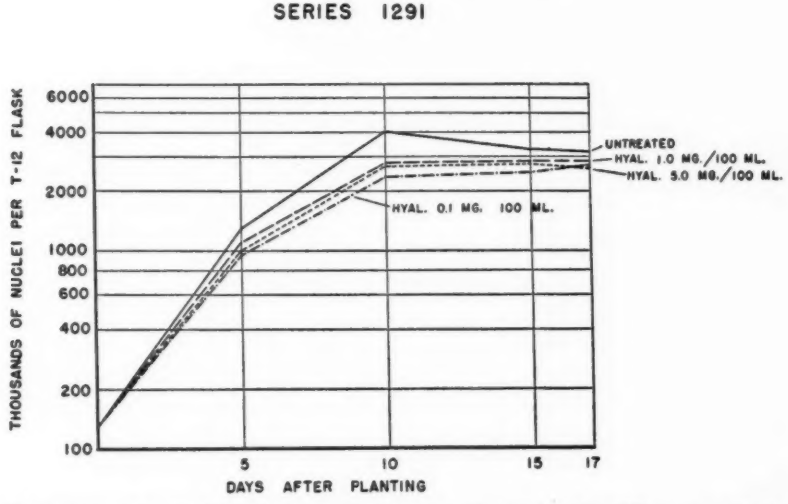
‡ The first lot of embryo extract for each treatment was used for the planting and the first two fluid changes; the second lot was used for the remaining fluid changes

§ See table 2, footnote ‡.

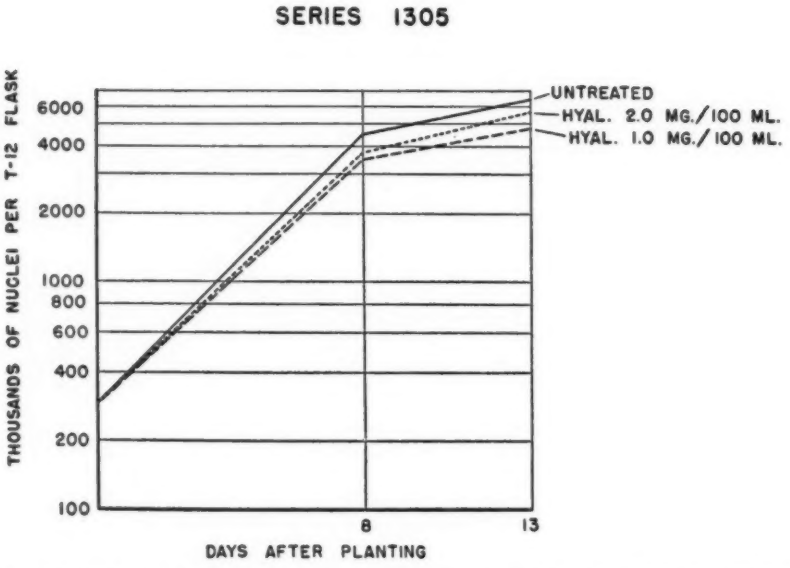
‖ The known nitrogen content and the apparent viscosity of the extract of lot 1493 were both unusually high, so it is probable that the period of ultracentrifugation was insufficient to bring about complete filtrability.



TEXT-FIGURE 5.—Proliferation curves of tissue cultures of series 1254 over a 10-day period. Hyaluronidase was present in the embryo extract for two groups at final concentrations of 0.1 and 1.0 mg. per 100 ml., respectively.



TEXT-FIGURE 6.—Proliferation curves of tissue cultures of series 1291 over a 17-day period. Hyaluronidase was present in the embryo extract for three groups at final concentrations of 0.1, 1.0, and 5.0 mg. per 100 ml., respectively.



TEXT-FIGURE 7.—Proliferation curves of tissue cultures of series 1305 over a 13-day period. Hyaluronidase was present in the embryo extract for two groups at final concentrations of 1.0 and 2.0 mg. per 100 ml., respectively.

cultures showed that the morphologic characteristics of the cells in the treated cultures of all three series were not noticeably different from those of the cells in the untreated control cultures (figs. 2 and 3). From these three experiments it appears that the action of hyaluronidase on the culture medium changes it so that it is only slightly less growth-promoting than is the medium in which the embryo extract is not treated with hyaluronidase. This slight difference in growth-promoting activity appears to be correlated directly with the total nitrogen concentrations, as may be seen in table 10. The four concentrations of hyaluronidase tested ranged from 0.1 to 5.0 mg. per 100 ml. The optimum proliferation rate was shown when the concentration of hyaluronidase in the embyro extract was 2.0 mg. per 100 ml.

Longer-term tissue culture experiments with four cell strains

The results of the short-term nuclei-count experiments seemed to warrant the use of filtered embryo extract on several cell strains over longer periods. Four cell strains routinely maintained in this laboratory were selected for the test.[16] The weekly preparation of about 100 ml. of filtered embryo extract was carried out according to the procedure indicated in table 10. Each week the same lot of embryos was used for both the treated extract and the corresponding untreated control extract. Data pertinent to this experiment are shown in table 11. While prolifer-

[16] This work was carried out by Miss Gwendolyn D. Likely, whose co-operation in this study is greatly appreciated.

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TABLE 11.—*Response of four cell strains in D-3.5 flasks to hyaluronidase treatment of the chick-embryo extract used in the fluid media*

Type of cell	Number of flasks		Duration of test			Extent of growth at close of experiment	
			Transfer generation no.		Time		
	Untreated	Treated	Start	End	(*days*)	Untreated	Treated
Strain L mouse fibroblast (*10*) #929	4	4	79	96	149	Excellent	Excellent
Mouse fibroblast (*15*) #1293	4	4	7 to 12	18 to 26	149	Excellent	Excellent
Mouse liver (*16*) #721	4	4	128	147	149	Excellent	Excellent
Chicken fibroblast #1336	4	4	5 to 8	11 to 13	89	Poor	Poor

ation of cells in these cultures was not determined quantitatively, no consistent difference in condition and rate of proliferation between the treated and the untreated cultures could be detected during the test period of 149 days. Three strains of mouse cells showed essentially the same growth response to hyaluronidase-treated embyro extract over extended periods of time that strain L had shown in the preceding nuclei-count study. The fourth cell strain tested, chicken fibroblasts, gradually became necrotic and finally was discarded at 89 days; the decline was apparently unrelated to any hyaluronidase effect, being similar in both treated and untreated cultures.

Longer-term tissue culture experiments with many cell strains and large lots of embryo extract [17]

Beginning October 19, 1951, filtered hyaluronidase-treated embryo extract was used regularly in the media for about half of all the cultures maintained in this laboratory. The method of preparation of this extract will be outlined in the following section. Certain strains of cells were carried entirely on media containing this filtered extract. The effect of hyaluronidase treatment of embryo extract on many different kinds of cells in tissue culture has thus been subjected to considerable further evaluation.

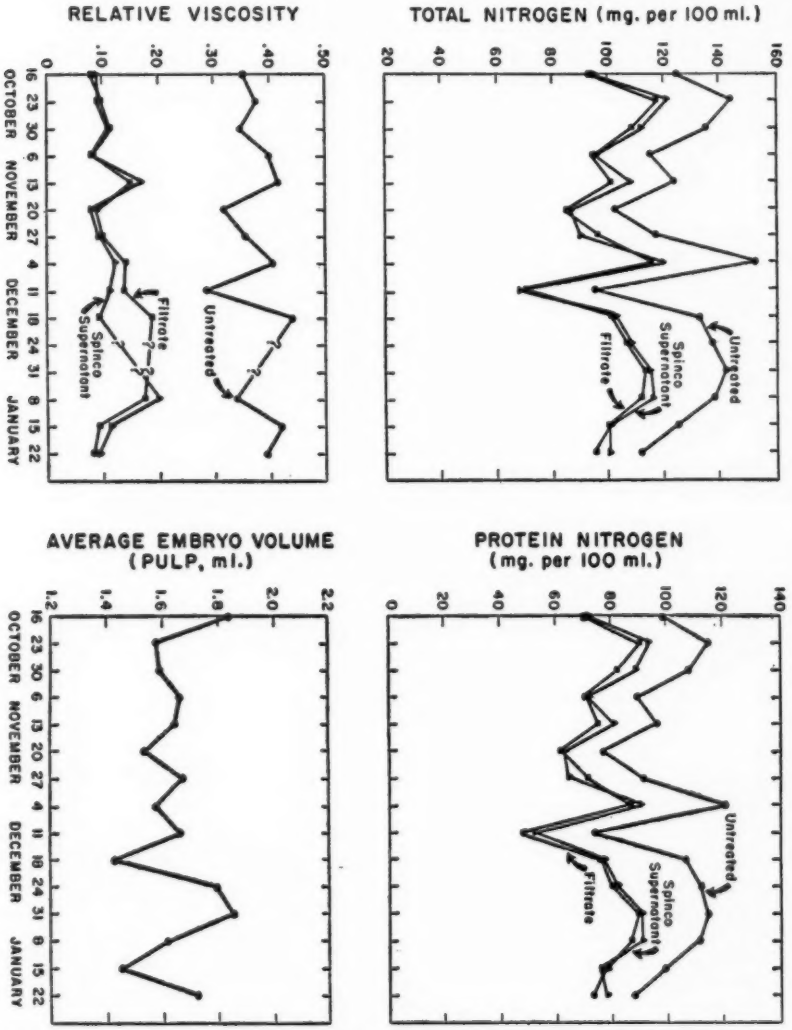
In this final large-scale test, filtered chick-embryo extract induced much the same growth of various cell strains in tissue culture as it had in the earlier tests. No substantial or consistent differences in condition and rate of proliferation have been detected between the treated and the untreated cultures. Consequently, on March 14, 1952 all tissue cultures in this laboratory were transferred to media containing filtered embryo extract; production of the old type of aseptically prepared extract was discontinued.

Trends in Characteristics of Extracts Prepared in Large Lots

The trends of total nitrogen, protein nitrogen, and specific viscosity at three stages in the process of preparation, and the average size of the 9-day embryos used for 15 consecutive weekly lots of filtered embryo extract are shown in text-figure 8. The average nitrogen content and average specific viscosity of the last 12 of these lots, prepared at a dilution ratio of 1:1.25, are shown in table 12. The striking variability in nitrogen content and in specific viscosity from one lot to another bears out long-recognized general observations on chick-embryo variability. Ultracentrifugation accounts consistently for nearly all of the nitrogenous compounds lost during preparation, while filtration itself causes little if any loss. All of the reduction in viscosity obviously occurs, on the average, before filtration. It has been shown above that the major part

[17] This phase of the investigation has depended to a great extent on the co-operation of other workers in this laboratory. Mr. E. L. Schilling has contributed to the development and application of the final procedure outlined below for the routine preparation of large amounts of filtered embryo extract. Miss Gwendolyn D. Likely, Miss Constance J. Mackey, Mr. John E. Shannon, Jr., and Miss Helen K. Waltz have maintained the tissue cultures and made the observations on the effects of filtered extract on the cells.

of this reduction is brought about almost immediately by hyaluronidase alone rather than by the subsequent ultracentrifugation. Variation in average embryo size from one lot to another has no relation to the variation in either nitrogen content or viscosity of the extracts.



TEXT-FIGURE 8.—Trends of total nitrogen, protein nitrogen, and specific viscosity of 15 consecutive weekly lots of filtered embryo extract at three stages in the preparation procedure, and average size of the 9-day-old chick embryos used for the extracts. The size of the embryo is calculated at the volume of pulp obtained per embryo in each lot.*

*Note added in proof: For "relative viscosity" in this chart read "specific viscosity."

TABLE 12.—*Average nitrogen content and average specific viscosity of 12 consecutive lots of 1:1.25 chick-embryo extract*

	Total nitrogen (*mg./100 ml.*)	Protein nitrogen (*mg./100 ml.*)	Specific viscosity*
Untreated extract	124.1	98.4	0.38
Supernatant from ultracentrifuge	100.5	76.4	0.11
Filtrate	99.0	75.2	0.12

*Ten consecutive lots.

FINAL ROUTINE PROCEDURE FOR PREPARATION OF LARGE LOTS OF FILTERED CHICK-EMBRYO EXTRACT

The method finally adopted and the one currently in routine use for the preparation of hyaluronidase-treated, ultracentrifuged, filtered chick-embryo extract is outlined here. Nine-day-old chick embryos are removed from eggs and placed in thick-walled 30 × 280 mm. pyrex test tubes. The tubes are immediately packed in powdered CO_2 ice to retard chemical changes and partially shatter the cells. After the whole lot has been collected and frozen the tubes are placed in water at about 37° C. or slightly below. As soon as the embryos have thawed they are ground in a specially designed Potter-Elvehjem type of grinder (text-figure 9) at about 400 r.p.m., grinding each lot through three or four up-and-down cycles.[18] The homogenizer cylinder has a maximum capacity for efficient grinding of about 85 nine-day-old chick embryos at one time (about 135 ml. volume, or 1.6 ml. each); the cylinder is then about two-thirds full. The pulp is withdrawn through the stopcock into a 2,000-ml. glass-stoppered cylinder and is diluted by adding 1.25 volumes of Earle's saline (*2*).[19] The mixture is centrifuged in an International No. 3 refrigerated centrifuge for 30 minutes at 2,300 r.p.m. (1,600 × g). The supernatants are pipetted into a 2,000-ml. graduated cylinder. A 13-ml. sample of the supernatant is taken for nitrogen analyses and viscosity determinations.

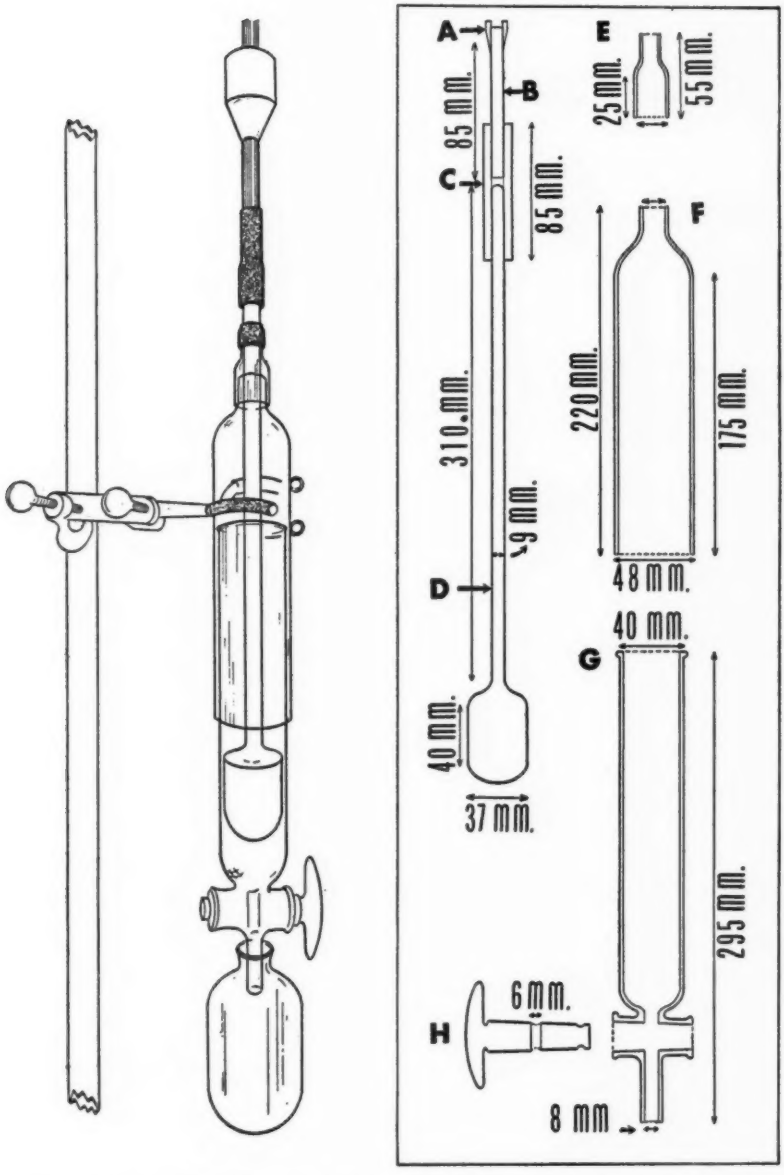
Hyaluronidase solution containing 1 mg. hyaluronidase per ml. is prepared without filtration immediately before use in order to avoid the possibility of a change in its viscosity-reducing activity. The hyaluronidase solution is added to the supernatant in the amount of 2 ml. per 100 ml., so that the final concentration of hyaluronidase in the embryo extract is approximately 2 mg. per 100 ml. The treated extract is mixed thoroughly and transferred to 120-ml. Lusteroid tubes.[20]

The extract is ultracentrifuged in a Spinco Model L preparative centrifuge at 20,000 r.p.m. (43,000 × g) for 150 to 180 minutes, with refrigeration set at about 2° C. Acceleration to maximum speed and deceleration from maximum speed to final stop each require about 25 minutes with a full #20 rotor, so that the total elapsed time for the ultracentrifugation alone is nearly 3½ to 4 hours. At the end of the run the supernatants are poured from the tubes into a graduated 2,000-ml. cylinder; the pellet is not disturbed by the pouring. A second 13-ml. sample of the pooled extract is taken for nitrogen analysis and viscosity determination. The empty

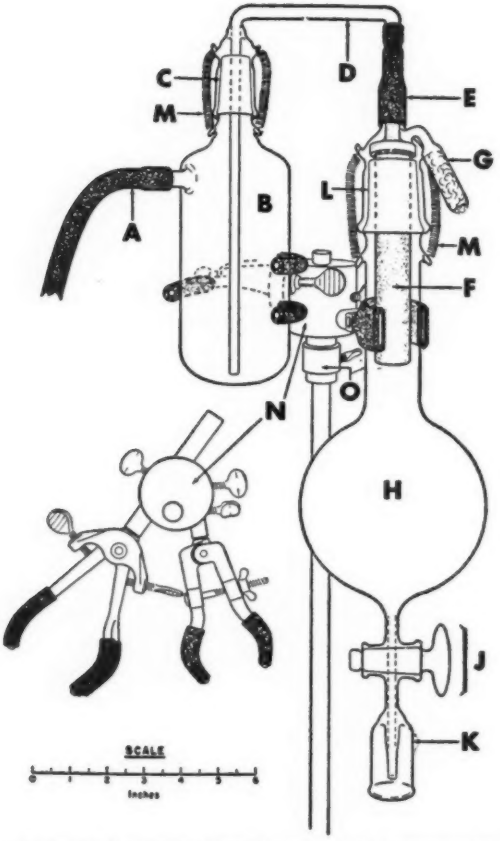
[18] It has been found by Schneider and Hogeboom (unpublished data) that the degree of cell disruption with a Potter-Elvehjem homogenizer depends more on the speed at which the cylinder is moved up and down by the operator than on either the clearance between the pestle and the cylinder walls or the speed of rotation of the motor-driven pestle.

[19] This 1:1.25 dilution ratio has been chosen in place of the 1:1 ratio in order to compensate approximately for the increase in total nitrogen found to result from the use of this homogenizer.

[20] These tubes are immersed overnight in triple-distilled water. It has been found that occasional collapse of Lusteroid tubes during ultracentrifugation is avoided by this soaking. The temperature during this soaking is held at about 3° C. to prevent bacterial growth.



TEXT-FIGURE 9.—Tissue homogenizer for grinding embryos. *A*, chuck attached to stirring motor; *B*, metal shaft; *C*, heavy-walled rubber tubing; *D*, pyrex pestle and rod; *E, F*, pyrex shields, to be used only for sterile extraction procedures; *G, H*, pyrex grinding cylinder with stopcock for drawing off pulp. Ground-glass surfaces on sides and bottom of both pestle and cylinder except for stopcock opening.



TEXT-FIGURE 10.—Improved pressure filtration unit. *A*, heavy-walled rubber tubing from gas tank; *B*, 500-ml. pyrex heavy-walled centrifuge bottle for pressure reservoir, with fused-in pressure inlet tube; *C*, pyrex ground joints, standard taper interchangeable, 24/40, with glass hooks; *D*, heavy-walled pyrex tubing, 9 mm. O. D., 3 mm. bore; *E*, surgical latex rubber tubing, to join pyrex tubing to connector of filter candle *F* and to upper end of ground joint *L;* this rubber tubing is bound tightly with heavy cord in order to prevent leaks; *F*, Selas filter candle, #03 porosity; *G*, pyrex tube packed with cotton for escape of air from collecting flask and sealed in to female part of ground joint *L; H*, collecting flask modified from 1000-ml. round-bottom pyrex flask; *J*, delivery stopcock, 4–5 mm. bore; *K*, sealed-on pyrex sleeve to protect delivery tip from contamination; *L*, pyrex ground joints, standard taper interchangeable, 40/50, with glass hooks; *M*, stainless steel springs; *N*, special Duralumin block, $2'' \times 2''$, for holding shortened laboratory clamps in fixed position; *O*, metal ring for additional support of block *N*. Pressure bottle *B* is surrounded by a metal safety shield when the unit is in use. In later work with filtration of embryo extract it was found that the use of a single rubber connector at *E* might possibly permit fluid to be forced directly from the pyrex tube *D* into the collecting flask *H*, thus by-passing the filter and permitting contamination. Therefore the single connector *E* was replaced by two adjacent rubber connectors, one joining tube *D* to the connector of the filter, the other holding the sleeve of pyrex joint *L* to the connector of the filter.

rotor and cover are washed immediately in order to avoid bacterial growth. The pooled extract is sterilized immediately by filtration through pressure filtration units (text-figure 10), allowing no more than 450 ml. of extract for each 1×6-inch filter. A third 13-ml. sample of the filtrates is taken for nitrogen analyses and viscosity determination. Tubes of filtrate are placed in the sub-zero cabinet and frozen. No further centrifugation is necessary when the tubes are thawed for use, because the thawed extract is clear.

Filtered embryo extract is currently prepared only once each week. The maximum number of embryos that can be processed in one lot is limited to the 1,200-ml. capacity of the large rotor of the ultracentrifuge. This volume requires approximately 475 nine-day chick embryos. The average time required for one-half this volume to pass through a Selas #03 filter, 1½×8-inch size, is 20 minutes.

Discussion

The practically complete passage of nitrogenous components of ultracentrifuged hyaluronidase-treated embryo extract through Selas porcelain filters was in marked contrast to the drastic reduction in nitrogen content that occurred during passage through a Berkefeld "N" filter in experiment 10. Similar adsorption of nitrogen has been reported by Davidson and Waymouth (*17*) in filtering untreated sheep-embryo extract through large Berkefeld candles. The 1:1 saline extract they used was viscous and tended to clog the filters easily; the relatively small amount of sterile filtrate obtained contained only 60 percent of the nitrogen that had been present in the extract before filtration. Carrel (*18*) found that chick-embryo extracts filtered through a Berkefeld filter always lost a great part of their power to activate the growth *in vitro* of chick connective tissue. From these observations it appears that thick-walled diatomaceous earth filters are not well suited for the filtration of embryo extract.

Selas porcelain filters have proved satisfactory not only in this investigation but also during six years of use in this laboratory for sterilizing various kinds of biological fluids. They have seemed particularly suitable for the filtration of tissue culture media because of certain characteristics: 1) The filter has no metallic parts; the filter element and the headpiece are porcelain, the porous candle being glazed directly to the glazed headpiece. 2) The filters can be obtained in an extended range of porosities and are reasonably uniform within any one porosity grade, even in different sizes.[21] 3) The filter can be cleaned thoroughly by acid digestion and firing so that there has been no cumulative clogging or other recognizable change in porosity after repeated use. 4) While the filter wall has mechanical strength to withstand the pressures used, the relative thinness of the wall reduces loss of substances from solutions being filtered. 5) Flow rate is rapid even when solutions such as serum or hyaluronidase-treated ultracentrifuged embryo extract are being filtered. 6) The uniform bacterial retentivenesss and maximum pore size of the #03 porosity filter are such that no instance of infection of culture media attributable to filter leakage has occurred in this laboratory.

In this investigation the hyaluronidase used reduced the viscosity of chick-embryo extract to a low level. It appears that this reduction in viscosity can be attributed to depolymerization of hyaluronic acid and related compounds. It has been found (*19, 20*) that the uniform structure of alternating acetylglucosamine and glucuronic acid residues which comprise hyaluronic acid is broken down by testicular hyaluronidase into compounds of considerably lower molecular weight, although not so far as to liberate monosaccharides. It seems probable that the molecular weights of the breakdown products from the hyaluronic acid in hyaluronidase-treated chick-embryo extract are likewise comparatively low. In such case some of the breakdown residues would remain in the supernatant during ultracentrifugation instead of being thrown

[21] We are informed by Mr. Jordan of the Selas Company that they can now furnish commercially filters of uniform wall thickness in any of the sizes. From our experience with the filtration of embryo extract, wall thicknesses of 3.0 to 3.5 mm. are preferred at the present time. Filters with thinner walls have shown some tendency to crack during incineration, while ones with thicker walls are avoided because of increased adsorption surface.

down in the pellet, and the nitrogen and phosphorus in the supernatant would be correspondingly higher. However, this tendency to leave more nitrogen and phosphorus in the supernatant would be offset at least in part by the lessening of the viscosity of the solution by hyaluronidase treatment, with consequent increase of the tendency for finely particulate matter to be thrown down in ultracentrifugation. While the question of whether or not hyalruonidase treatment should precede ultracentrifugation merits further study, it has been decided to standardize the method tentatively and add hyaluronidase to the extract before ultracentrifugation. Since the hyaluronidase-treated extract does allow rapid proliferation of the cells, when used with serum, it appears that the original highly polymerized material broken down by the enzyme is not essential for proliferation of the cells. Nor did the products of this depolymerization show any demonstrable toxicity under the conditions studied.

Tennant, Liebow and Stern (*21*) found some growth-promoting activity in the residue fraction of ultracentrifuged embryonic extract. Waymouth [22] has evidence of a semiquantitative nature that the particles centrifuged out from chick-embryo extract at high speeds played some part in promoting proliferation of tissue cultures in an otherwise synthetic medium. The pellet obtained at 100,000 × g for 60 minutes appeared less active than that obtained at 10,000 × g for 30 minutes, but the data did not indicate what proportion of the activity of the whole extract was thus removed.

In the present study the growth-promoting effect of the residual pellet from ultracentrifuged chick-embryo extract has not been tested. The present work does show that the filtered supernatant from ultracentrifugation, after hyaluronidase treatment and combination with horse serum and saline, is extremely active in promoting cell proliferation and can be used satisfactorily for the routine maintenance of tissue cultures. The fact that many cultures of numerous cell varieties were still proliferating as actively at the end of five months as at the beginning of the test indicates that the filtered supernatant would continue to promote active proliferation for indefinitely extended periods of time.

An early hope that hyaluronidase alone could be depended upon to allow filtration has not been realized. To date, even clarifying filtration following hyaluronidase treatment has not allowed the elimination of ultracentrifugation before filtration. It thus seems clear that ultracentrifugation and hyaluronidase are mutually essential.

Davidson and Waymouth (*22*) grew fresh explants from 9-day embryo chick heart for one or two days in a 50:50 medium of Tyrode's solution and 9-day chick-embryo extract mixed with mucinase solution. A highly purified preparation of mucinase from bull testicle was used in a series of final concentrations from 50 to 350 mg. per 100 ml. Treated cultures showed a smaller increase in nucleoprotein phosphorus than did the controls, particularly at mucinase concentrations greater than 170 mg. per 100 ml. The mucinase remained active in the roller tubes, so it was thought possible that this inhibiting effect might be due to a direct action on the cells rather than an action on the embryo extract. They concluded that mucopolysaccharides may be responsible for at least some part of the growth-promoting power of embryo extract. In their work, however, the concentrations of mucinase were all much greater than any of the concentrations of hyaluronidase used in the present study. The hyaluronidase concentration in embryo extract necessary for the degree of mucopolysaccharide inactivation that will permit filtration is only 2 mg. per 100 ml. No evidence of hyaluronidase toxicity to tissue cultures has been found in the present study.

In the present study the hyaluronidase-treated extract was used in conjunction with serum in studies on the proliferation of the cells. Serum is known to exercise a strong inhibiting action on hyaluronidase (*6*). In this respect too, the present studies on the behavior of the hyaluronidase-treated extract differ from the studies of Davidson and Waymouth; serum was not added to their culture media and the mucinase was demonstrably active in the cultures. Until more data are available, caution is advisable in the use of hyaluronidase in culture systems where serum or another comparable inhibitor does not inactivate the hyaluronidase.

[22] Personal communication March 18, 1951.

At the present time, therefore, chick-embryo extract prepared by the methods described cannot be considered as interchangeably equivalent to extract prepared without hyaluronidase, ultracentrifugation, and filtration. Use of the filtered extract on cell types other than those studied or under other experimental conditions should be undertaken cautiously until its characteristics are more fully known. In those systems where filtered embryo extract can be used, however, its advantages greatly recommend its use, most especially in large-scale culture operations.

This study has been limited to extracts from chick embryos of 9 to 10 days' incubated age. Further experimental work is needed to determine whether the method finally adopted is applicable without change to embryos of greater age or of other species.

Three short-term tissue culture experiments, evaluated quantitatively by the nuclei-count method, gave preliminary data indicating that hyaluronidase in ultracentrifuged and filtered chick-embryo extract in the nutrient media was not toxic to strain L cells and allowed essentially comparable proliferation levels as did untreated extract. These data have been fully validated by the more extensive tissue culture studies here reported. Use of this filtered extract during five months in the routine maintenance of large groups of cultures of a number of cell strains has proved practical and satisfactory. All cell strains tested have appeared to proliferate as well in media containing filtered extract as in media containing extract prepared with rigid asepsis.

This filtered extract has been used for several months in the media for single isolated tissue cells in culture. It has proved to be considerably more uniform and reliable than was the aseptically prepared extract formerly used. This application to single-cell studies is considered to be a most critical test of the nutritional capacities of the extract.

In earlier attempts to isolate and maintain pure cell strains there has always been the possibility of contaminating the strains with living cells carried over into culture from the embryo extract in the nutrient media. This hazard has persisted even though various precautions such as clarification or freezing of the extract have been used. Four pure strains of cells have already been grown from isolated single cells. With each of these strains a glass or cellophane substrate is regularly used for the luxuriant growth of the cells. The serum and balanced saline constituents of the nutrient medium are now sterilized by filtration. The present study makes practical the routine filtration of large amounts of highly active embryo extract. Consequently pure strains of cells can now be maintained in culture for indefinitely extended periods of time without hazard of contamination of the strain by tissue cells from the medium.

This method of preparation and sterilization of extract now permits the preparation of larger lots of embryo extract with less expenditure of effort and time, and with greater uniformity of product than was possible with the older sterile procedure. A major factor in the saving of time is the rapidity of the filtration process: one liter of treated extract can pass a 1½×8-inch Selas #03 filter in about 30 minutes. It is now possible to carry the extract through various procedures of chemical and physical manipulation with an accuracy heretofore unobtainable because sterilization is effected as the final step in preparation. The method seems to be well adapted for the preparation of large lots of embryo extract such as would be required in large-scale commercial production.

Summary

The characteristics of various filters and their suitability for the filtration of chick-embryo extract and other tissue culture media have been examined. Special Selas #03 porosity filters were used for most of the investigation. The filters used and the methods and equipment for using, testing, and cleaning the filters are described.

Two major factors were found to interfere with filtrability of extract from 9-day-old chick embryos. The first, finely particulate material, was thrown out from the solution by ultracentrifugation. The second

factor appeared to be hyaluronic acid and related compounds. This factor was eliminated by treatment with a low concentration of hyaluronidase for short intervals.

Nitrogen in the extract decreased considerably during ultracentrifugation but showed no further appreciable decrease during filtration of the ultracentrifuged extract.

There was no evidence that either hyaluronidase or hyaluronic acid breakdown products were injurious to the cultures tested; nor was there evidence that they retarded cell proliferation under the experimental conditions used.

A procedure has been developed for preparing large amounts of chick-embryo extract for use in tissue culture. Nine-day-old chick embryos were frozen in dry ice; these were thawed immediately and were ground in a motor-driven tissue homogenizer. The pulp was extracted with 1.25 volumes of Earle's saline. After low-speed centrifugation of the mixture the supernatant was treated with hyaluronidase to give a final concentration of approximately 2 mg. of enzyme per 100 ml. of extract. The treated extract was ultracentrifuged 2½ to 3 hours at 43,000 × g. The supernatant was filtered under pressure through special Selas #03 filter candles and frozen. No further centrifugation was required when the extract was thawed for use.

As many as 475 nine-day chick embryos, representing approximately 1,200 ml. of extract, may easily be processed in each lot, this volume being the capacity of the ultracentrifuge used. This volume, subdivided into two lots, easily passes through the filters in less than 20 minutes under 8 to 12 pounds of pressure.

Three short-term quantitative tissue culture experiments with strain L mouse cells of single-cell origin showed that filtered embryo extract in the media gave proliferation rates substantially as high as did untreated extract. Five-month tests with many cell strains in several types of flask cultures under both filtered and untreated extract showed no discernible difference in growth and condition of tissue cultures. The filtered extract was extremely active in promoting cell proliferation. This type of extract is now in satisfactory use on all cultures being maintained in this laboratory.

The serum and balanced saline constituents of the nutrient medium are now sterilized by filtration. The present study makes practical the routine filtration of large amounts of highly active embryo extract for this medium. Consequently pure strains of cells can be maintained in culture for indefinitely extended periods of time without hazard of contamination of the strain by tissue cells from the medium.

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PLATE 19

FIGURE 1.—Filter candles, cut open to show variations in wall thickness:
A, Selas #03 porosity, 2×10-inch size;
B, Selas #03 porosity, 1½×8-inch size;
C, Selas #03 porosity, 1×6-inch size;
D, Selas #03 porosity, ⅝×2-inch size;
E, Mandler fine porosity, 2×10-inch size;
F, Mandler fine porosity, 1×5-inch size;
G, Mandler regular porosity, ⅝×2-inch size;
H, Berkefeld "N" porosity, 1×5-inch size.

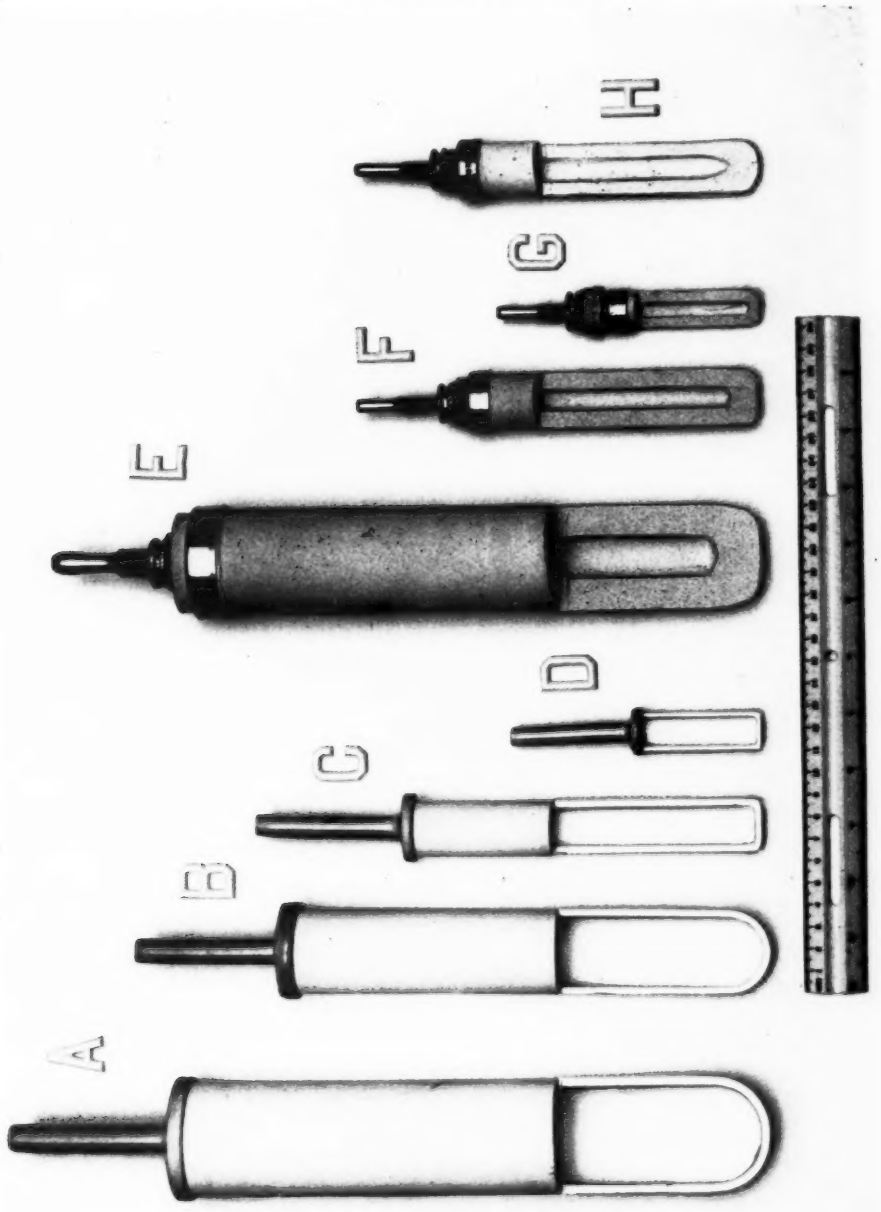

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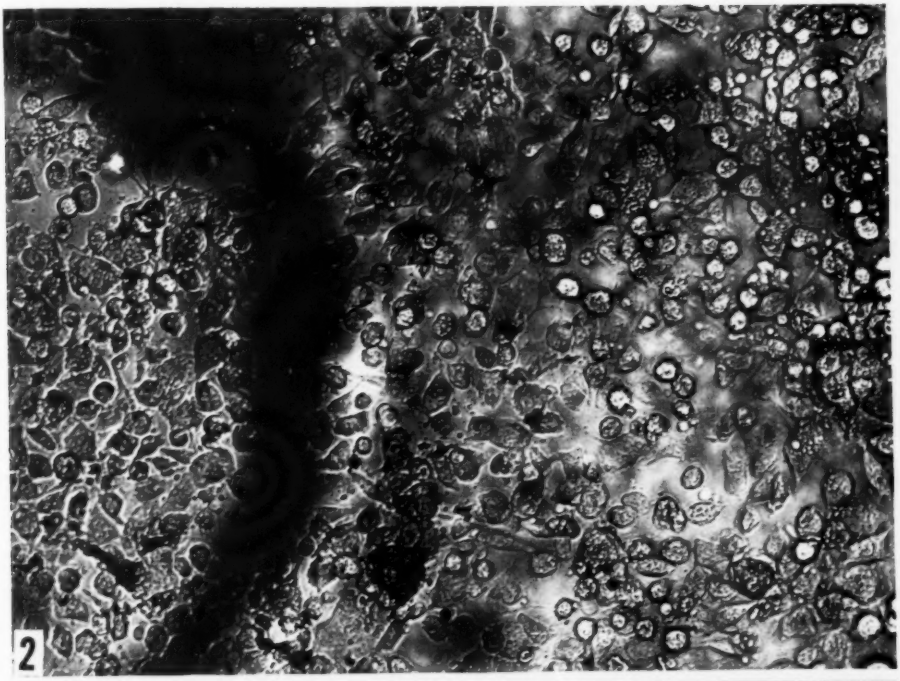
PLATE 20

FIGURE 2.—Strain L mouse cells growing under perforated cellophane in T-12 flask. Series 1254 at 10 days, control, with untreated aseptically prepared chick-embryo extract. × 200

FIGURE 3.—Strain L mouse cells growing under perforated cellophane in T-12 flask. Series 1254 at 10 days, experimental, with filtered chick-embryo extract that had been ultracentrifuged and treated with hyaluronidase at a final concentration of 1.0 mg. per 100 ml. of extract. × 200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ANNOUNCEMENT

Establishment of "Transplantation Bulletin"

Within recent months efforts have been under way to increase the exchange of information in the field of tissue transplantation among investigators in laboratories and those at the bedside. The USPHS sponsored a small conference which took place in October 1952 at Arden House, Harriman, New York. At this conference workers in plastic surgery, cancer, zoology, and other fields discussed common problems in tissue transplantation. In March 1953, the Ciba Foundation sponsored a similar conference in London, England, which emphasized "The Preservation of Normal Tissues for Transplantation." Participants at both conferences felt that some means should be provided for further continuous exchange of information on an informal basis among investigators in the clinic and the laboratory interested in problems of tissue transplantation.

To meet this need, it is proposed to issue a quarterly "Transplantation Bulletin." This Bulletin will serve several functions:

1) It will maintain, and publish at least once yearly, a Transplantation Registry which will list all practicing physicians and research workers, both in the United States and abroad, interested in transplantation problems. The Registry will cover the fields of plastic surgery, endocrinology, cancer, genetics, immunology, experimental morphology, etc.

2) The Bulletin will provide a medium for a rapid and informal exchange of information on problems and progress in the clinics and laboratories both here and abroad. It is emphasized that the Bulletin will not publish formal papers. However, brief comments on unreported data will be welcomed.

3) It will, through a staff of Corresponding Editors, attempt to keep all members of the Transplantation Registry informed of forthcoming meetings of the professional societies at which there will be presentations dealing with any aspects of transplantation. It will also try, through its Corresponding Editors, to have the subjects covered in a coordinated fashion at the meetings.

4) It will maintain a bibliography in the fields listed in item (1) above.

The Transplantation Bulletin hereby extends an invitation to all workers in the fields of medicine and biology interested in tissue transplantation, to submit their names and fields of interest to E. J. Eichwald, M.D., University of Utah College of Medicine, Salt Lake City, Utah. A subscription fee of $3.00, to cover the expenses of printing the Bulletin and maintenance of the Registry and bibliography, will be payable after the first issue of the Bulletin has appeared. The first issue is expected to appear in August 1953.

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Applications for Grants in Cancer Research

The Committee on Growth of the National Research Council, acting for the American Cancer Society, is accepting applications for grants-in-aid in support of cancer research. Applications for new grants received before October 1 will be considered during the winter and grants recommended at that time will become effective July 1, 1954. Investigators now receiving support will be notified individually regarding application for renewal of these grants.

The Committee feels that a clear understanding of cancer must rest upon a deeper insight into the nature of the growth process, normal and malignant. Therefore, the scope of the research program is very broad and includes, in addition to clinical investigations on cancer, fundamental studies in the fields of cellular physiology, morphogenesis, genetics, virology, biochemistry, metabolism, nutrition, cytochemistry, physics, radiobiology, chemotherapy, endocrinology, and environmental cancer.

During the past year the American Cancer Society, on recommendation of the Committee on Growth, has awarded approximately 250 grants totaling more than $1,700,000. A program of similar magnitude is contemplated for the coming year.

Application blanks and additional information may be obtained from the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, Washington 25, D. C.

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SUGGESTIONS TO AUTHORS

1. Follow a recent copy of the Journal of the National Cancer Institute as to style, especially in regard to tables, illustrations, references, abbreviations, etc.

2. Submit an original and two carbon copies of the manuscript in order to expedite handling and review. Use a good grade of paper, approximately 8 by 10½ inches. Type all material double spaced.

3. The Style Manual of the United States Government Printing Office and Webster's New International Dictionary (latest edition) are followed in matters of spelling, capitalization, and hyphenation. Dorland's Medical Dictionary and Chemical Abstracts are followed for medical and chemical terms when they are not found in Webster. The Quarterly Cumulative Index Medicus is followed in abbreviation of periodical titles.

4. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

5. Number each page. Begin each page with a new paragraph; that is, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This regulation is required by the Government Printing Office.

6. Insert each footnote in the text immediately after the line in which it is mentioned.

7. Type each table on a separate sheet of paper. Insert each table as the page immediately following the first reference to it. Tables should be referred to in the text and should be numbered in sequence.

8. Halftone illustrations (photomicrographs and photographs) are printed as plates at the end of each article. Mount all photographs on heavy cardboard, as many together as will make one 5¼- by 8-inch plate. Legends for plate-figures should be typed on separate sheets of paper. Linecut illustrations (graphs and charts) are printed in the text and are designated as text-figures. Legends for text-figures should be typed in the text (set off by lines across the page) immediately following the first place the text-figure is mentioned. All legends should be typed double spaced with a two-space hanging indention; that is, all lines except the first should be indented two spaces. One set of illustrations is sufficient. The maximum size of illustrations is 5¼ by 8 inches.

9. Designate all halftones as Figures 1, 2, 3, etc., continuously from plate to plate throughout manuscript. Submit graphs in final form. Write the figure number lightly on the back of each illustration and indicate the top of each.

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